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Influence of Nutrients on Developmental Programming of Glucose and Lipid Metabolism in a Wistar Rat Model

by

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Abstract

Objectives: To determine the effects of pre-natal diets high in protein or prebiotic fiber on offspring health in adulthood and to examine the potential for postnatal prebiotic fiber to mitigate the metabolic malprogramming associated with maternal protein restriction during pregnancy. **Methods:** Three main studies were performed. Maternal diets high in prebiotic fiber (21.6%; HF) or protein (40%, HP) were compared with control. Gut microbiota and milk were analyzed in the dams along with adult offspring body composition, plasma satiety hormones and gut microbiota after a high fat/sucrose (HFS) dietary challenge. Re-matching to maternal diet after the HFS challenge was examined in the second study. The HF diet was further examined as a weaning diet in offspring malprogrammed due to gestational protein restriction (PR) in the third study. **Results:** Two oligosaccharides were increased in maternal milk of HP and HF dams. HF dams also had increased levels of bifidobacteria compared to HP and C. In the offspring, HF diets decreased body weight and adiposity, and increased plasma PYY and the abundance of bifidobacteria in the gut across all studies. The HP diet increased offspring body weight and adiposity, but decreased HOMA-IR scores. Liver triglycerides were also increased, which in the second study was accompanied by upregulation of hepatic lipogenic genes (SREBP1c, ACC, FAS). In the first two studies HF decreased plasma lipopolysaccharide, a measure of metabolic endotoxemia. Re-matching to maternal diet blunted the typical response to the HF and HP diets with increased fasting glucose, energy intake and decreased bone mineral density seen in re-matched versus naïve offspring. In the final study with malprogrammed offspring, HF improved HOMA-IR scores in male offspring but also increased markers of intestinal permeability in female PR offspring. **Conclusions:** A maternal diet high in prebiotic fiber prevented excessive weight and adiposity gain in adult offspring during an HFS dietary challenge. While improving HOMA-IR scores, the HP diet lead to increased adiposity and increased hepatic lipogenesis. A weaning diet high in prebiotic fiber has beneficial effects on body weight and adiposity, plasma satiety hormones, glycemia and gut microbiota in malprogrammed offspring.

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Dedication

To my family.

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List of Symbols, Abbreviations and Nomenclature

Symbol	**Definition**
ACC1	Acetyl-coenzyme A Carboxylase alpha
ACN	Acetonitrile
AIN	American Institute of Nutrition
AMPK	AMP-activated protein kinase
ANOVA	Analysis of variance
AUC	Total area under the curve
B4GALT6	Beta-1,4-galactosyltransferase
BCL10	B-cell CLL/lymphoma 10
BMD	Bone mineral density
BMI	Body mass index
C	Control
C1	Offspring of dams fed control diet
CC	Offspring consuming control of dams fed control
CD14	Cluster of Differentiation 14
CF	Offspring consuming HF of dams fed control
CpG	Cytosine phosphate guanine
CPN2	Carboxypeptidase N, polypeptide 2
CT	Threshold cycle
D	Diet
DIO	Diet-induced obese
DPP	Dipeptidyl peptidase
DXA	Dual energy x-ray absorptiometry
ELISA	Enzyme-linked immunosorbent assay
F	Fiber diet
FA	Formic acid
FAS	Fatty acid synthase
FITC	Fluorescein isothiocyanate
FOS	Fructo-oligosaccharide
G6Pase	Glucose-6-phosphatase
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GC	Gas chromatography
GDM	Gestational diabetes mellitus
GIP	Gastric inhibitory polypeptide
GK	Glucokinase
GLP-1	Glucagon-like peptide-1
GLP-2	Glucagon-like peptide-2
GLUT1	Glucose transporter 1
GLUT2	Glucose transporter 2
GLUT5	Glucose transporter 5
H	Rats fed HFS from 14-28 weeks of age
HF	High fiber

HF1	Offspring of dams fed high fiber diet
HFS	High glucose/sucrose
HMO	Human milk oligosaccharides
HOMA-IR	Homeostatic model of assessment (insulin resistance)
HP	High protein
HP1	Offspring of dams fed high protein diet
HPA	Hypothalamic-pituitary-adrenal
HPLC	High performach liquid chromatography
IBS	Irritable bowel syndrome
ICAM2	Intercellular adhesion molecule-2
IGF-I	Insulin-like growth factor-1
IGF-II	Insulin-like growth factor-2
IgG	Immunoglobulin G
IL-1α	Interleukin 1 alpha
IL-6	Interleukin 6
IUGR	Intrauterine growth restriction
IVGTT	Intravenous glucose tolerance test
LC	Liquid chromatography
LGALS2	Lectin, galactoside-binding, soluble-2
LPS	Lipopolysaccharide
MPP	Mass Profiler Professional
MS	Mass spectrometry
MTII	Alpha-melanocyte-stimulating-hormone analog
Muc2	Mucin 2
NEFA	Non-esterified fatty acid
NPY	Neuropeptide Y
OGTT	Oral glucose tolerance test
OFS	Oligofructose
OS	Oligosaccharide
PCA	Principle component analysis
PEPCK	Phosphoenolpyruvate carboxykinase
PGC-1α	PPAR-γ coactivator-1 alpha
PI3K	Phosphoinositide-3-kinase
POMC	Proopiomelanocortin
PPAR-γ	Peroxisome-proliferator-activated receptor-gamma
PRC	Protein restricted offspring consuming control
PRF	Protein restricted offspring consuming fiber
PTPRCAP	Protein tyrosine phosphatase, receptor type, C-associated protein
PYY	Peptide tyrosine tyrosine
Q-TOF	Quadrupole-time-of-flight
R	Reference animals

RF	Reference fiber animals
RP	Reference protein animals
RT	Retention time
RT-PCR	Real-time polymerase chain reaction
S	Sex
SCFA	Short chain fatty acid
SEM	Standard error of the mean
SGLT-1	Sodium linked glucose cotransporter-1
SNAT2	Sodium coupled neutral amino acid transporter-2
SREBP1c	Sterol regulatory element binding protein-1c
Tff3	Trefoil factor-3
TJP1	Tight junction protein-1
TNF-α	Tumor necrosis factor-α
TNFSF13	Tumor necrosis factor ligand superfamily member-13
WAP	Whey acid protein
wt	Weight
ZO-1	Zonula occludens-1

Chapter One: Introduction

1.1 Background

Environmental exposures encountered in the periods surrounding pregnancy and early childhood influence the development of every individual. Mothers supply all the nutrients necessary for a developing embryo and fetus, and thus the health status of and environment created by the mother has a profound impact on the health of the fetus. This maternal influence can have effects lasting throughout the offspring's life and can even be passed on to future generations[1].

The transmission of disease risk from mother to offspring may in large part be due to the phenomenon called "programming". Programming is defined by Lucas[2] as "an induction, deletion or impaired development of a somatic structure resulting from a stimulus or insult during a critical period that has long-term consequences for function". Nutritional programming is the concept that offspring react to cues about the environment, created by the mother's nutritional experience and make changes in growth and metabolism aimed at improving survival after birth based on these cues[3]. A poor nutritional environment *in utero* has been implicated in programming increased susceptibility to obesity, cardiovascular disease, hypertension and type 2 diabetes[2]. Programming has been shown to affect birth size and maternal weight of future generations, even up to twelve generations later[1, 4].

The chronic diseases affected by programming can be difficult and costly to treat. The economic costs, direct and indirect, associated with obesity in Canada in 2001 were estimated to be $4.3 billion[5]. Today approximately 60% of Canadian adults and 32% of children are classified as overweight or obese and the costs have risen accordingly[6]. More recently the rate of increase in the number of overweight and obese Canadians has slowed, which is promising, but in fact means that there remain a very high number of Canadians living with an unhealthy body weight and those of child-bearing age have the potential to influence future generations. Treatment strategies such as diet and exercise are poorly adhered to, and if and when weight loss is achieved, maintenance of the

weight loss is notoriously difficult. Prevention of obesity, therefore, has been identified as a strategy of utmost importance[7, 8].

Maternal weight gain during pregnancy has a far-reaching impact on the health of mother and infant, with excessive weight gain leading to increased risk of maternal weight retention after delivery and of having a high birth weight infant (>4000 grams)[9, 10]. Excessive weight gain is also associated with gestational hypertension and augmented labour, and in morbidly obese women is associated with increased risk of neonatal metabolic abnormality[11]. A high birth weight is also a risk factor for becoming overweight in adulthood[9]. Lowell and Miller[12] recently showed that forty-one percent of Canadian women entering pregnancy with a normal BMI gained more weight than recommended during their pregnancy, and 55% of women with a BMI greater than 27.0 gained more weight than recommended. Simple and safe strategies to help manage weight gain during pregnancy and provide an optimal nutritional experience could have positive outcomes for maternal and infant health and future generations.

Numerous diets have been promoted for their ability to enhance weight loss and support weight maintenance. High protein diets, such as the Atkins Diet or South Beach Diet, have been popular and well marketed[13, 14]. These diets claim to enhance weight loss and reduce food intake by promoting the intake of higher levels of protein and very low levels of carbohydrate, in theory leaving an individual feeling fuller for longer. Scientific evaluation of high protein diets has in fact shown them to be effective for weight loss in adults, with multiple clinical trials showing enhanced weight loss over 6 months, as well as improvement in various components of the metabolic syndrome such as serum triglycerides and insulin sensitivity, compared to a low-fat diet[15-17]. A high protein diet has also been shown to be effective for treating women with polycystic ovarian syndrome[18].

A second component of dietary patterns that is often manipulated in weight loss diets is the fiber content. High fiber diets, particularly those with prebiotic fiber, such as inulin and oligofructose, have been shown to promote weight loss and enhance feelings of fullness, an effect that is likely due in part to increased production of the satiety hormones glucagon-like peptide-1 (GLP-1) and peptide tyrosine tyrosine (PYY)[19].

Diets high in fiber are also recommended for the prevention and treatment of numerous chronic conditions, including dyslipidemia and colorectal cancer, and have even been recommended for the prevention of preeclampsia[20, 21]. The impact of consuming diets high in fiber or protein during pregnancy and lactation, however, has not been extensively studied but merits attention due to the potential influence they exert at critical periods in embryonic and fetal development. Evidence from animal studies utilizing a high protein diet during pregnancy has largely shown adverse outcomes, with offspring body weight being negatively impacted[22]. A maternal prebiotic fiber diet has been shown to influence offspring gut microbiota profiles but further effects have not been widely reported[23].

The gut microbiota has been well documented for its effects on host health, including body weight and metabolism[24]. More recently the gut microbiota have been linked to inflammatory conditions through compromised gut barrier function and the subsequent presence of the endotoxin lipopolysaccharide (LPS) in the blood[25]. Changes in the microbiota composition during pregnancy have also been reported, as well as similarities between mother and child at different ages[26]. Prebiotic fiber has been repeatedly shown to improve bacterial profiles in the gastrointestinal tract[25, 27], while diets high in protein have been shown to have negative effects on microbial communities[28-31]. The high degree of similarity shown in the gut microbiota of dams and pups[32] suggests that improving the profile of maternal gut microbiota may improve early colonization of the offspring gut through bacterial transfer *in utero*, at birth, and during suckling[33, 34]. The suckling period appears to be of great importance for establishment of the neonate's gut microbiota as oligosaccharides present in maternal milk have non-nutritive benefits due to their fermentation by bacteria in the colon. These oligosaccharides can stimulate the growth of Bifidobacteria, a hallmark of breastfed infants and a bacterial species associated with numerous beneficial health effects such as improved gut barrier function, decreased inflammation and improved glucose tolerance[25]. Studies examining the relationship between maternal diet during pregnancy and lactation and the establishment of offspring gut microbiota, including the

influence of maternal milk oligosaccharide content, are needed to fully understand the transmission of disease risk from mother to offspring.

1.2 Significance of Study

This work was designed to examine the impact of specific maternal dietary components on offspring health and determine if there is an interaction between the diet consumed by rat dams during pregnancy and lactation and the diet consumed by the offspring postnatally. Examination of two key nutrients, namely protein and fiber, and their effects on maternal and offspring health will increase our understanding of how individual dietary components contribute to nutritional programming. Eventual translation of this animal work into human clinical studies has the potential to lead to improved dietary guidelines for pregnancy. If these early diets have inherent preventative potential for obesity, they could have far greater impact than treating overweight or obesity and the subsequent co-morbidities once established.

1.3 Objectives

The objectives of this work were to:

(1) Examine the role of a maternal diet high in prebiotic fiber or protein on excess weight gain and metabolic dysregulation in offspring in response to a high fat high sucrose diet in young adulthood.

(2) Examine whether re-matching offspring to the same diet consumed by their mothers during pregnancy and lactation reduces the negative effects of a high fat high sucrose diet challenge in adulthood.

(3) Examine the role of maternal diet and subsequent maternal milk composition in establishing lasting gut microbiota profiles in offspring and how these profiles differ along the gastrointestinal tract;

(4) Examine whether consumption of a high prebiotic fiber diet in offspring mitigates some of the detrimental effects associated with exposure to a maladaptive maternal protein-restricted diet during pregnancy.

1.4 Hypotheses

The 'Thrifty Phenotype Hypothesis' put forth by Hales and Barker[35] postulated that poor fetal and/or infant nutrition are detrimental to organ development and function. When this is paired with a drastic difference in nutritional experience in later life, or catch-up growth, the greater mismatch and accelerated growth result in increased risk for diseases such as type 2 diabetes or cardiovascular disease. We hypothesize that a high prebiotic fiber maternal diet or offspring weaning diet will result in a controlled rate of offspring growth and nutrient-gene interactions that provide protection against excessive weight and adiposity gain into adulthood. We hypothesized that many of these effects will be mediated by the gut microbiota. Conversely, a maternal diet high in protein will predispose offspring to excessive weight and adiposity gain. This work is specifically designed to examine the effects of diets high in prebiotic fiber on offspring health after exposure in early life, either during pregnancy and lactation or at weaning. It will also examine the effects of varying levels of protein during pregnancy.

1.5 Presentation

This thesis is composed of a number of chapters arranged in the following order: Chapter 1 is a brief summary of the topic including the significance, objectives and hypotheses; Chapter 2 reviews the relevant literature; Chapter 3 is a published manuscript summarizing the effects of maternal diets high in protein or prebiotic fiber on offspring body weight and composition, satiety hormones and glucose metabolism after a high fat high sucrose diet; Chapter 4 is a manuscript submitted for publication and describes the microbial communities of the dams consuming high protein or prebiotic fiber diets and their offspring, as well as the milk composition of the dams; Chapter 5 is a manuscript summarizing the effects of re-matching offspring to their respective *in utero* & lactation diets, high protein or prebiotic fiber; Chapter 6 is a manuscript submitted for publication summarizing the effects of weaning onto a high prebiotic fiber diet after exposure *in utero* to a maternal protein-restricted diet; Chapter 7 summarizes and discusses the findings of the previous chapters and suggests future directions for this research. While

Chapter 3 has been published, chapters 4 and 6 are submitted and currently awaiting a decision regarding acceptance for publication. Chapter 5 will be submitted shortly. Each manuscript consists of an introduction, methods and materials, results and a discussion. These chapters are supplemented by one appendix, Appendix A, that is a short communication exploring the impact of maternal diets high in prebiotic fiber or protein on the morphology of the colon of the male adult offspring.

Chapter Two: Literature Review

2.1 Introduction

Evidence coming from animal and human studies has shown a profound effect of the early environment on the health of offspring later in life. The influence of nutritional and non-nutritional factors on health due to exposure *in utero* and/or in the early postnatal period can impact risk of developing obesity, cardiovascular disease, metabolic syndrome and type 2 diabetes through a variety of proposed mechanisms.

2.2 Developmental Programming

Programming is a long-term physiological setting in response to an environmental change during a critical period[2]. It is believed an unfavourable prenatal environment may trigger adaptations aimed at improving fetal survival and preparing the fetus for the same predicted unfavourable environment it will encounter in postnatal life[36]. There is a substantial body of evidence describing fetal or developmental programming with influences including both nutritional and non-nutritional factors. A poor *in utero* nutritional environment has been implicated in programming later cardiovascular disease, hypertension and type 2 diabetes[2]. In relation to diabetes, offspring glucose metabolism has been shown to change as a result of maternal dietary manipulation[2]. This programming can affect birth size and maternal weight of future generations, even up to twelve generations later[1, 4].

2.2.1 Mismatch and Re-matching

It has been established that a mismatch between the pre- and post-natal environment can result in adverse effects in regards to appetite regulation and glucose and lipid metabolism[37]. Consistency in balanced pre- and post-natal nutritional environments may be associated with improved health compared to settings where animals experience a mismatch between these two periods[37]. A mismatch that occurs between pregnancy and lactation can also have adverse effects on offspring. For example,

pups exposed to an adequate or high protein diet during pregnancy had increased body weight when switched to the opposite diet during suckling compared to animals exposed to the same diet throughout pregnancy and lactation[22].

Aside from one example where male offspring from dams fed a high fat diet were fed the same diet at weaning and had decreased plasma triglycerides compared to controls and improved endothelial function compared to littermates given a control diet at weaning[38], a high fat maternal diet is an anomaly, where matching pre- and postnatal nutrition is not ideal. Many studies have shown that feeding a balanced diet at weaning, after exposure to a maternal high fat diet, results in a partial reversal of the detrimental metabolic effects of *in utero* high fat exposure[39-42]. Female rats weaned onto a high fat diet demonstrate hyperphagia and have increased fat pad mass, serum leptin and insulin levels[43]. If re-matched to a high fat diet in adulthood mice show increased susceptibility to the negative effects of a high fat diet including increased weight, larger adipocytes, glucose and insulin intolerance, mitochondrial dysfunction, adipocyte insulin resistance and hepatosteatosis[44]. The negative impact of a high fat diet appears reversible between pregnancies given observations that switching obese animals onto a low fat diet prevents the previously experienced adverse consequences of hyperphagia, adiposity, hypertension, insulin resistance, dyslipidemia and hepatic steatosis in the offspring even with continued maternal obesity[45].

In a low protein model, rats that were weaned onto the same protein restricted diet as their mothers had bone mineral density that was even lower than that of animals whose mothers only received the low protein diet; they also had greater body fat percentage[46]. It would thus appear that matching diets is only advantageous if the diet itself has recognizable nutritional benefit, however "healthful" diets have not been examined for their effects on offspring metabolic health when matched between maternal exposure and offspring exposure in adulthood.

2.2.2 Reversal of Programming

Once programming has occurred due to an adverse maternal environment, treatment interventions are necessary to mitigate the potential consequences for offspring

health. Vickers et al.[36] first attempted to reverse adverse programming associated with caloric restriction during pregnancy in rats using 10 days of leptin injections in neo-natal female offspring. At 170 days, animals that received early post-natal leptin injections rather than saline did not experience excessive weight or fat gain in response to a hypercaloric diet and were in fact normalized to the values of control rats[36]. Locomotor activity, food intake, fasting plasma leptin, C-peptide, and insulin were also normalized in leptin treated animals. The authors then attempted to replicate the findings in male rat pups, but with measurements taken at 110 days[47]. There was no effect of leptin treatment on food intake in the male rats when fed a hypercaloric diet, however, when fed a standard chow diet, leptin treatment did decrease fat mass in conjunction with decreased insulin and C-peptide compared to untreated animals. More recently growth hormone has been used to treat neo-natal rats from 3-21 days of age. Administration of growth hormone resulted in increased insulin sensitivity in treated versus untreated males derived from undernourished dams[48].

In one of the first studies to specifically examine the role of a dietary intervention in reversing programming due to maternal undernutrition, Burdge et al.[49] provided a folic acid supplement in post-natal diet for 4 weeks, followed by 4 weeks of high fat feeding. Folic acid supplementation, regardless of maternal diet, resulted in increased body weight, hepatic triglyceride content and plasma triglycerides in offspring at 84 days of age. While supplementation of the offspring with folic acid did not normalize offspring of protein-restricted mothers to the offspring of control dams, it did demonstrate that there is plasticity in the metabolic systems of weanling rats that may, with the appropriate intervention, allow for “resetting” an inappropriate phenotype. Recently, a successful postnatal nutritional intervention was reported in a model of maternal glucocorticoid excess in the second half of pregnancy[50]. The characteristic increase in blood pressure, adiposity, plasma triglycerides and cholesterol in offspring of this model was worsened with consumption of a high fat weaning diet, but prevented by supplementation with omega-3 fatty acids, whether pups were weaned onto a control or high fat diet[50]. Other models of programming and post-natal intervention, particularly non-invasive dietary

treatments, still need to be examined for their potential role in improving offspring health.

2.3 Proposed Mechanisms of Developmental Programming

2.3.1 Epigenetics

A number of mechanisms have been proposed to explain observed programming effects, the main suggested mechanism being epigenetics. DNA methylation and histone modifications are changes in genes that occur without alteration to the DNA sequence itself and are referred to as epigenetic changes. DNA methylation occurs primarily on the cytosines of the dinucleotide sequence cytosine phosphate guanine (CpG)[51]. Methylation status at a single locus can result in a large difference in offspring phenotype as evidenced by the agouti mouse. A methyl-supplemented diet during pregnancy results in fewer offspring exhibiting the yellow coat obese syndrome, and more brown-coloured, lean offspring[52]. In humans, DNA methylation of certain genes has also been examined in the context of the chronic diseases associated with the Dutch Famine[53] as well as in the context of increased adiposity in children[54].

In addition to DNA methylation, histone modification can also contribute to epigenetic changes. Histones allow the packaging of DNA into nucleosomes to form chromatin. The structure and function of chromatin can be changed via post-translational modifications to the histone which in turn impacts transcriptional activity or DNA repair[51]. Deacetylation of histones can result in changes in transcription factors that lead to disease. For example, deacetylation of histones H3 and H4 from intrauterine growth restriction results in silencing of Pdx1 and decreased insulin production in pancreatic ß cells[55].

2.3.2 Placenta

Some programming effects occur at the organ level and appear to be largely independent of epigenetic changes. The placenta acts as a mediator between mother and

fetus and is a potential means of achieving developmental programming through changes in its function or morphology impacting transport of maternal factors to the fetus. The development of this organ takes place during the first trimester and may be an originator for the sex-specific effects observed in developmental programming as its size and environmental adaptation depends on the sex of the embryo[56]. These sex-specific differences are also seen in gene expression, which is divergent between the sexes and also dependent on maternal diet[57, 58].

Diet plays an important role in development and function of the placenta. A low protein diet has been found to up-regulate placental genes involved in apoptosis and growth inhibition, and down-regulate expression of genes governing nucleotide metabolism, insulin signalling and amino acid transporters[59, 60]. Increased nutrient transport via glucose transporter 1 (GLUT1) and sodium coupled neutral amino acid transporter 2 (SNAT2) can also occur with a high fat diet in mice resulting in large fetuses[61]. A high fat, high sugar diet (HFS) in mice has different effects depending on the stage of pregnancy. On day 16 of pregnancy the most profound differences due to diet were observed: decreased weight of the gravid uterus, increased transfer of glucose and amino acids per gram of placenta with an even greater increase in accumulation in the fetus, decreased volume of the maternal decidua basailis, decreased volume of fetal capillaries, increased trophoblast interhemal membrane thickness, increased expression of imprinted genes controlling fetoplacental growth and allocation of maternal resources (though only one gene showed minor changes in DNA methylation), and decreased insulin receptor abundance though downstream elements of the insulin signalling pathways were upregulated[61]. At day 19 many of these effects were no longer evident although signalling through the PI3K pathway was still increased. It is suggested that the effects seen at day 16, such as accumulation of nutrients in the fetus, can result in the adverse effects observed later in life[62].

2.3.3 Tissue-Level Changes

Most tissues in the body show evidence of programming due to changes in maternal environment. Energy may be spared from development of organs deemed less

important for survival in order to preserve those that are absolutely necessary such as the brain[4]. As a result there may be decreases in nephron number in the kidney[63], reduced pancreatic β-cell number, size, mass and proliferation[64, 65] and increased β-cell apoptosis[66]. This β-cell apoptosis may occur due to increased sensitivity to cytokines[37].

The ability to maintain homeostasis and the 'setting' of homeostasis may also be affected in numerous tissues. In the liver, a maternal low protein diet can result in increased glucose production and suppression of enzymes involved in lipid homeostasis, while a high fat diet increases fetal hepatic apoptosis[67-69]. Peripheral tissues are also affected by maternal nutrient restriction, with skeletal muscle exhibiting signs of insulin resistance[70] and adipocytes accumulating more lipid due to upregulation of IGF-I and IGF-II receptors[71].

Developmental programming may also occur from the remodelling of the hypothalamus and other brain regions[72]. The neuroendocrine regulatory systems of the hypothalamus can be affected from disturbances that are hormonal, metabolic, or nutritional[73]. Evidence has been found from both over- and undernourished models for changes in hypothalamic pathways and function[73-77]. When ewes were over-fed during late gestation, their offspring had increased pro-opiomelanocortin (POMC) receptor mRNA expression in the arcuate nucleus of the hypothalamus, without a corresponding decrease in food intake[77]. The over-feeding of these ewes during a period of rapid fetal growth resulted in a failure of hypothalamic anorexigenic pathways to be upregulated in response to increased adiposity, likely due to central resistance to the actions of leptin, with resultant obesity[77]. It has also been found that maternal exposure to an HFS diet results in changes in the central reward system that increase fat intake at 3 months of age in rats[75].

In a model of under-nutrition, Plagemann[73] used a low protein diet throughout pregnancy and lactation which resulted in disorganization of the main hypothalamic regulators of body weight and metabolism, and malformed hypothalamic nuclei in offspring. Malprogrammed adult rats show changes to hundreds of genes in the hypothalamus, with two clusters related to insulin signalling and nutrient sensing and the

detection and use of lipids for fuel being greatly affected[76]. More recently a decreased food intake response to neuropeptide Y (NPY) was shown in protein restricted rat pups along with an increased response to the anorexia-inducing melanocortin 3/4 receptor agonist MTII[74].

2.4 Evidence from Human Studies

In the 1970s the seminal observations for the evidence that early life exposures influence adult health were reported. In Holland, adults born after the Dutch Hunger Winter (1944-1945) had unique health consequences based on the timing of their prenatal exposure to famine. Data collected from 19 year old conscripts found that obesity was higher when exposure to maternal undernutrition occurred during the first half of gestation than in the last trimester or immediately after birth[78]. In 50 year old females, maternal famine exposure during early gestation with adequate food availability in late gestation was associated with increased body weight, BMI and waist circumference, which was also associated with birth weight[79]. When asked to recall their body weight at age 20, BMI was reportedly higher in males due to early famine exposure, but higher in females with late exposure. With exposure to famine during mid to late gestation, offspring were lighter, shorter and thinner at birth and subsequently had a lower glucose tolerance at age 50, with an effect that was not solely related to the famine, but also to birth size[79]. Another consequence of birth size unrelated to the famine was increased blood pressure with decreased birth size[79].

Following the initial observations surrounding maternal undernutrition, birth size was also shown to impact disease risk, with low birth weight increasing risk of heart disease[80], glucose intolerance and type 2 diabetes in a United Kingdom cohort[81]. High birth weight is also related to increased health risks later in life as numerous studies relate high birth weight and later overweight status, which increased risk of metabolic syndrome, cardiovascular disease, and type 2 diabetes[9]. Infant birth weight may be increased as a result of excessive gestational weight gain, maternal obesity or gestational diabetes[9]. Excessive gestational weight gain may impact offspring through mechanisms beyond increased birth weight such as changes in appetite control, neuro-endocrine

pathways, adipose tissue development and energy metabolism[82]. Similarly, gestational diabetes mellitus is associated with numerous adverse outcomes for offspring at birth and into adulthood including macrosomia, birth trauma, overweight or obesity, metabolic syndrome and type 2 diabetes[83].

2.5 Evidence from Animal Studies

Evidence for the programming effect in a variety of animal models is extensive. A compromised nutritional environment is one of the chief triggers of malprogramming and as such, numerous different diet compositions have been examined for their effects on offspring health. First to be examined were diets involving restriction, whether from reduced energy or deficits in protein intake. In more recent years, examining diets of excess has been of increasing relevance due to the increased consumption of diets high in fat or protein in the western world.

2.5.1 Programming due to Maternal Low Protein

One common restrictive model is that of intra-uterine growth restriction (IUGR). IUGR can occur due to factors related to the fetus or placenta, which in turn are dependent on or influenced by maternal diet and other environmental factors[84]. Protein restriction (8% protein by weight as opposed to the 20% required for normal growth) has been widely used as a model for intrauterine growth restriction and will be the focus of this section of the literature review. The measureable effects of this maternal diet seem to have differential effects based on age and sex, as well as timing of the exposure to the low protein diet. Some effects shown early in life have been found to be reversed as animals reach an advanced age. For example, when dams consumed a low protein diet during pregnancy and lactation, male offspring had decreased fasting plasma glucose and insulin at 6 weeks of age compared to controls and a smaller rise in glucose in response to an IVGTT. These animals also had smaller fat pads and decreased adipose cell number and size[85]. Male offspring were smaller than controls throughout their lives (up to 17 months), at which point they exhibited high fasting plasma glucose, peak glucose during

an IVGTT, glucose AUC, and fasting insulin and AUC[86], a complete reversal of observations from 6 weeks of age. In females of protein restricted dams, glucose tolerance has been shown to be similar to controls up to 21 months of age, although fasting insulin and AUC were significantly higher indicating decreased glucose uptake and emerging insulin resistance[87].

When protein restriction occurred selectively during pre- or post-natal life, fasting plasma glucose in offspring at 6 weeks of age was not different from controls, although fasting insulin concentrations were lower, as was the rise in blood glucose during the IVGTT[85]. Regardless of whether low protein restriction occurred pre- or postnatally, animals had smaller fat pads than controls, although animals exposed prenatally had larger fat pads than animals exposed only in the postnatal period. Size of adipocytes for both groups was intermediate, being larger than animals exposed to protein restriction during both periods, but smaller than controls, demonstrating that there was some recovery that was not completely dependent on weight as only prenatal low protein animals showed recovery of body weight during the study. Similar to low protein animals, prenatally exposed animals had twice as many insulin receptors in adipocytes[85]. Food intake in animals examined out to 100 days of age was lower with suckling exposure to a protein restricted diet[88]. Post-natal exposure has been shown to result in higher whole-body protein content in males and females, while whole-body lipid content was decreased at 70 days of age; this being accompanied by lower fasting glucose at 110 days of age[88].

In mice with prenatal protein restriction, body weight and composition was not different from controls, although females had decreased insulin sensitivity when exposed to a high fat diet from 6 to 20 weeks of age[89]. A study using rats found that females, but not males, had lower birth weights after protein restriction and at 21 days both sexes had decreased weight regardless of maternal diet during the lactation period[88]. Males had higher concentrations of triglycerides and cholesterol versus controls[88]. Both males and females of protein restricted dams had higher diastolic blood pressure and those exposed to a high-fat diet had a higher heart rate[89]. In pigs exposed pre-natally to low protein, birth weight was lower but catch up growth occurred so that by weaning there

was no significant difference in weight from controls. Development of the small intestine was impacted as at birth villi length was longer compared to controls, though this effect did not last[90]. At 188 days small intestine muscularis thickness was less than controls[90].

Collectively the current literature provides solid evidence for the broad scope of effects of a maternal low protein diet during pregnancy and/or lactation and the long-lasting consequences for offspring health.

2.5.2 Programming due to Maternal High Fat

In regards to nutritional excess, the high fat diet has likely been examined to the greatest extent. A maternal high fat diet appears to exert different effects based on numerous factors related to the mother and the diet. For example, the high fat diet can be accompanied by maternal obesity although not necessarily, and/or decreased glucose tolerance and insulin sensitivity[45]. The fatty acid composition of the high fat diet, whether high in saturated fat, or specific polyunsaturated fatty acids, can also influence the effects on the offspring[45].

Most frequently a high fat diet during pregnancy has been found to increase birth weight of offspring, resulting in later metabolic syndrome, or components thereof. Exposure to a high fat diet *in utero* has been associated with increased insulin resistance, liver weight, hepatic steatosis, visceral fat mass, adipocyte hypertrophy, serum tumor necrosis factor α and interleukin 1ß, blood pressure, and bone marrow adiposity[91-95]. Some of these increases, such as blood pressure, can be seen as early as 13 weeks of age[94]. Pathways involved in glucose and lipid metabolism are also specifically influenced with the expression of numerous genes being altered by a maternal high fat diet[91, 96]. In the hypothalamus a maternal high fat diet has been found to increase mRNA levels of genes associated with disturbed appetite regulation including neuropeptide Y, Agouti-related peptide, pro-opiomelanocortin and melanocortin receptor-4. Orexigenic peptides have also been found to increase in the paraventricular nucleus and perifornical lateral hypothalamus[97].

Offspring sex, along with weaning diet, also plays a role in susceptibility to these detrimental metabolic outcomes following maternal high fat diet. Female offspring demonstrate increased cholesterol levels regardless of fat content of their weaning diet, whereas male offspring show these increases only when consuming a high fat diet. Resultant blood pressure measures also demonstrate a sex effect with females exposed either *in utero* or during suckling to high fat diet having increased blood pressure, while males did not differ from controls[38]. Conversely, another study showed some protection for female offspring after a maternal high fat diet as they had decreased hepatic triglyceride content accompanied by potential upregulation of fatty acid oxidation in the liver[98]. Opposite effects of a high fat diet have also been seen in male and female offspring in regards to hepatic PGC1α mRNA and resultant HOMA-IR scores; males but not females being negatively impacted by the maternal high fat diet[99]. Given the sex-specific effects demonstrated in the literature to date, there is clear justification for the inclusion of both male and female offspring in future studies.

2.6 Effects of a Maternal High Protein Diet

In addition to the ubiquitous availability of high fat foods in western cultures, high dietary protein intake is also of relevance to the current work. High protein (HP) diets, referring to a diet of 40% protein by weight, have been popularized for individuals trying to lose weight due to their purported satietogenic and thermogenic effects[100]. People following a high protein diet for a short time lose weight and have improved glycemic control, although this may be a result of the reduced body weight[101]. Over a longer period of time, however, high protein diets have been associated with impaired glucose metabolism and type 2 diabetes[101, 102]. In rodents, consumption of a high protein diet causes the animals to eat less and lose fat mass, whereas pair-fed animals lose mass more uniformly from all organs[103].

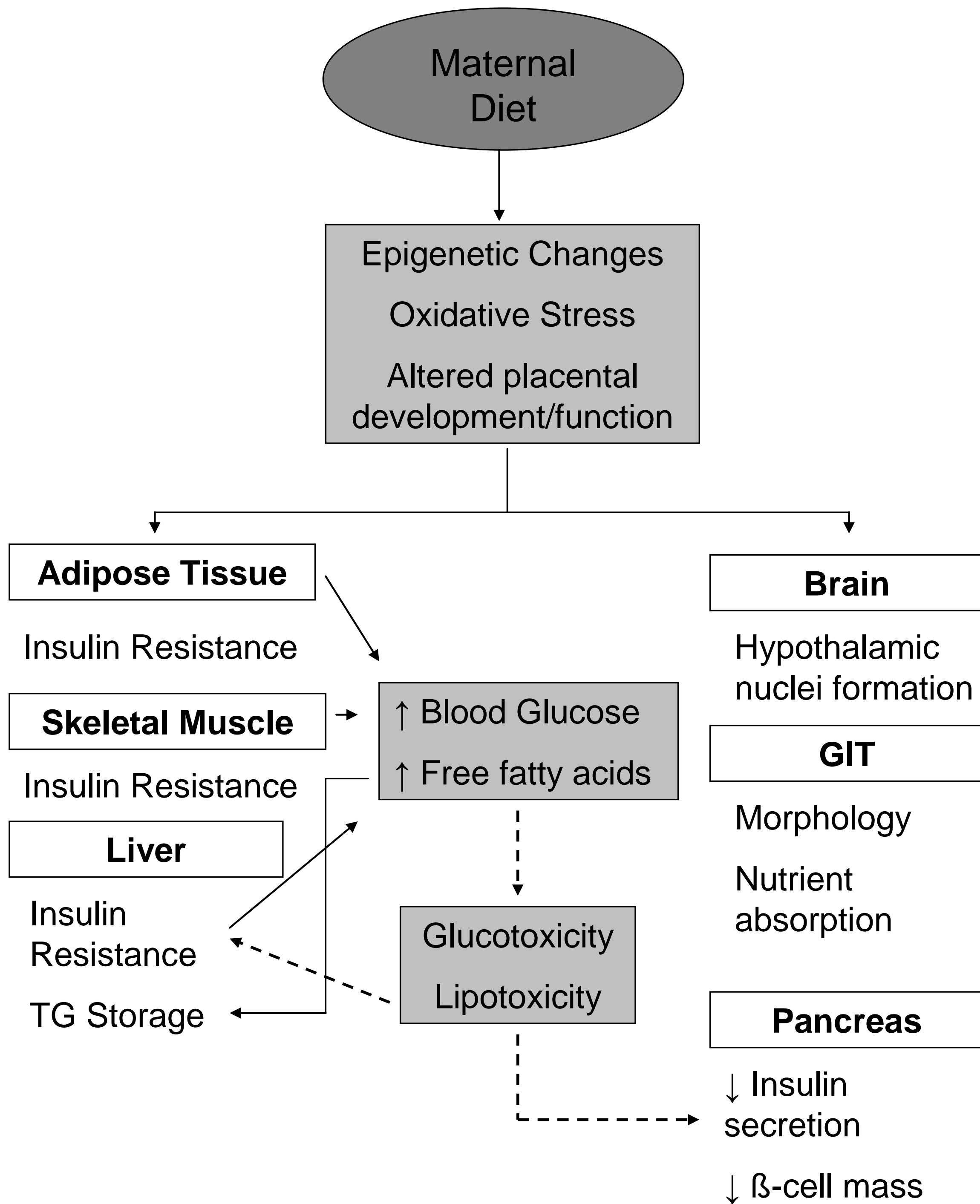
Protein intake is also high in various populations that are not trying to lose weight. A recent study examining diet during pregnancy found that some pregnant women consume up to 350 g of protein per day[104]. High protein diets have also been found to be effective in treating polycystic ovarian syndrome[105], and it is therefore

likely that consumption of this diet would continue into pregnancy in these women. Most evidence indicates that a high protein diet consumed during pregnancy and/or during the lactation period can have detrimental effects on the health of the offspring, having a long term effect on various tissues, and even offspring survival[106]. Findings in regard to offspring body weight in response to a high protein diet during pregnancy have not been consistent. In humans it has been found that with increased protein to carbohydrate ratios, and when an increased percentage of macronutrients is derived from protein during pregnancy, fetal abdominal visceral area is increased[107]. There is also one report of a high protein supplemented diet during pregnancy being associated with children born small for gestational age and although body weight at one year of age was not different, there were differences in some measures of psychological development[108]. Birth weight was also negatively associated with third trimester protein intake in women self-reporting their diet using a Food Frequency Questionnaire[104]. Adult offspring of women consuming a diet high in animal protein had increased blood pressure, as well as cortisol levels, which has been linked to smaller placentae[109, 110]. In formula-fed infants, it has been found that a high protein formula results in faster growth, which has been associated with later obesity. At 2 years of age, weight and weight-for-length was greater for infants receiving a high-protein formula, despite similar energy intakes with the low- and high-protein formulas[111]. Similarly, rat pups weaned onto a high protein diet display increased body weight, adiposity and glycemia after consuming a high fat and sucrose diet for 6 weeks in early adulthood compared to controls[112, 113].

Further research in rodent models has shown that male rats exposed to an HP diet *in utero* had decreased birth weight and animals switched from or to a dam consuming HP at birth had higher body weight after weaning[22]. Pre-natal exposure to an HP diet resulted in increased fat mass and decreased total energy expenditure in rats although resting metabolic rate was not affected[22]. In animals, it has also been found that exposure to a maternal HP diet during lactation decreases body and muscle growth in offspring due to decreased muscle protein accretion. This change was associated with a shift towards a preference for oxidative metabolism[106]. Sows fed a HP diet during pregnancy produced offspring that had a lower birth weight and lower body weight to

188 days of age[90]. These piglets also had longer villi in the small intestine at birth and lower intestinal saccharase activity at 188 days[90]. Others have not seen differences in birth weights but by 22 weeks female rats from HP dams have increased body weight, as well as increased ovarian fat pad mass at study termination, while males did not differ from controls[114]. Food intake was shown to be greater in HP females post-weaning, although reduced after puberty so that cumulative food intake was lower, indicating increased food efficiency in HP females[114]. While there were no significant differences in body weight, HP males did have higher blood pressure at 4 weeks of age through to 22 weeks[114].

Figure 2.1. Effect of maternal diet on offspring organ development and function



2.7 Effects of Dietary Fiber

Dietary fiber is an important and often overlooked component of the diet. It is recommended that adults consume 20 to 38 g per day[20]. Fiber decreases the energy density of the diet and habitual fiber intake is associated with decreased fat intake and increased food volume[115], as well as lower body weight[116].

Inulin and oligofructose belong to a class of fiber called prebiotic fibers which are non-digestible carbohydrates that selectively stimulate the growth and/or activity of certain bacteria in the colon to improve host health[27]. Inulin is a long chain fructo-oligosaccharide usually derived from chicory root while oligofructose is a shorter chain fructo-oligosaccharide derived from inulin. A diet high in prebiotic fiber has been shown to have beneficial effects on satiety, food intake, hypercholesterolemia and glycemic control, weight loss, fat mass reduction and the abundance of bifidobacteria in the gut[117, 118]. Beneficial effects on intestinal growth and function have also been observed[112, 119]. Within this thesis a high fiber diet is considered one which contains at least 10% fiber by weight, up to 21.6% fiber by weight.

Prebiotics have been widely investigated in infant nutrition for their potential to improve immune function in relation to atopic diseases, particularly in formula-fed infants. Animal models showed prebiotic supplementation increased vaccination response and decreased allergic reactions[120]. In humans, prebiotics are associated with a decreased number of infectious episodes, increased postvaccination IgG antibodies and decreased incidence of atopic dermatitis[120-122]. When consumed at weaning in animals, prebiotic fiber diets have been shown to decrease weight and adiposity gain, increase plasma GLP-1, and affect hepatic and brown adipose tissue gene expression[112, 113].

Epidemiological evidence showed increased insulin sensitivity in pregnant women with increased vegetable and fruit fiber intake and decreased risk of GDM with increased total, cereal and fruit fiber intake[123, 124]. Brazilian women have been found to have a very high fiber intake, which is associated with gestational weight gain within the normal range[125]. Women in the Brazilian study who did not reach the minimum

recommended daily intake of fiber had an increased risk of weight retention postpartum and obesity[125]. When consumed by rats during pregnancy, a fiber supplemented high fat diet increased placental weight and fetal number compared to control and high fat fed groups. Malformation and necrosis of placental trophoblasts due to the high fat diet were prevented, amino acid transporter genes had increased mRNA levels, and super-oxide dismutase activity in fetal liver was increased in response to the fiber[126]. A diet containing 21% prebiotic fiber by weight (1:1 ratio inulin and oligofructose) consumed by rat dams during pregnancy and lactation was associated with decreased liver weight, higher GLP-1 and higher glucose transporter expression in the intestine of offspring at 21 days[113]. A prebiotic supplemented diet including galacto-oligosaccharides and inulin has been found to increase pup growth through increased muscle mass[127].

Prebiotic fiber, however, has not had exclusively positive effects across all experiments. In populations with gastrointestinal disorders such as irritable bowel syndrome (IBS) and Crohn's disease, prebiotic fiber has not had the same bifidogenic effect as in healthy subjects[128, 129]. In IBS patients, initial supplementation with fructo-oligosaccharides was associated with worse symptoms than the placebo group[130]. Therefore caution is required when considering the effects of prebiotic fibers on populations with compromised gut function.

Prebiotics have had largely positive effects on health and have even shown promise for correcting parameters associated with poor maternal diet[126]. Further investigation on long term effects and corrective potential of this dietary component is needed.

2.8 Maternal Milk Composition

Malprogramming can occur as a result of compromised maternal nutrition during pregnancy but also during lactation when the offspring are completely dependent on maternal milk for their nutritional input. Differences in maternal milk composition seem to occur with drastic changes in macronutrient content of maternal diet. Dams fed a diet of 60% glucose produced milk with a higher fat content than dams fed a 12 or 24 % glucose diet; whereas dams fed a glucose-free diet had higher protein concentration and

lower fat and lactose concentrations in their colostrum[131]. With very large changes in maternal protein intake, as well as type of protein, changes in protein content of maternal milk have also been observed. When wheat gluten was used as the protein source in rats during lactation, milk collected from these dams had lower protein content than when casein was the protein source[132]. The higher quality casein diet produced milk that was higher in quality (i.e. higher in casein content) and a lower quantity of dietary casein was required to achieve similar milk levels of protein[132]. A high fat diet in obese mice was found to decrease the protein content of milk[133]. Interestingly, protein content of milk has been found to increase in mothers with anemia[134].

Findings on the effect of low protein maternal diets on the fat content of the milk have not been consistent. King et al.[135] found decreased fat content in the milk of sows fed a low protein diet. Pine et al.[136], however, found that rat dams fed a low protein diet during lactation had increased milk fat concentration on days 4 and 8 of lactation. It was also found that a low protein diet during lactation resulted in decreased milk lactose concentration by day 8[136]. Maternal high protein diets (42.6% wt/wt) have also been found to decrease lactose content of the milk, likely related to increased gluconeogenesis during lactation as a result of glycogen depletion in the liver from prolonged intake of the high protein, low carbohydrate diet[137, 138]. High fat diets have had mixed results as to their influence on milk composition. Diets high in lipids have been shown to increase fat concentration of maternal milk and also to increase daily milk volume[139]. However a diet high in corn oil, fed to mice only during lactation, was found to decrease milk fat content[140].

While differences in macronutrient composition appear to require large changes in maternal diet, fatty acid composition of maternal milk has been shown to be responsive to changes in maternal fatty acid intake. Changing the type of oil used in the diet has been shown to affect nervonic acid content in maternal milk which subsequently impacts the heart and liver of the offspring[141]. Supplementing the maternal diet with butter, margarine or olive oil resulted in fatty acid composition of the milk being similar to that of the fat supplement consumed by the dam, and also to having lower levels of saturated

fatty acids. These differences in milk occurred even with only small changes in maternal blood fatty acids[142].

Dietary changes resulting in differences in milk composition may at least partially be due to changes in mammary gland function. Glucose restriction, as well as protein and energy restriction, has been shown to decrease mammary gland cell size[131, 143]. Mammary mRNA abundance was found to be lower when a high protein diet was consumed during pregnancy, lactation, or both[137]. There appear to be numerous genes responsible for the production of each component of the milk, and can function at varying levels of mRNA abundance, as changes in expression observed with a high protein diet did not result in changes in milk fat or protein content as may have been expected given the changes in mRNA abundance. For example, consumption of a high protein diet decreased mRNA of α-lactalbumin by 80% while the concentration of lactose in the milk decreased by only 20%[137]. Obesity has been widely associated with increased difficulty in initiating breastfeeding and earlier cessation[144, 145]. In diet-induced obese mice it was found that on the day after birth, expression of several genes involved in milk synthesis including α-lactalbumin, ß-casein and whey acid protein (WAP) were all decreased[133]. At day 10 of lactation, ß -casein and WAP had recovered although α -lactalbumin was still greatly decreased compared to controls and associated with decreased milk volume[133]. These changes in gene expression were accompanied by morphological defects including a reduced branching frequency and impaired alveolar development in the mammary gland[133]. While changes in protein and fat composition of maternal milk have been examined in response to maternal diet, other chief components of milk, such as the oligosaccharides, which act as prebiotics in the infant gut have yet to be examined.

2.9 Microbiota

The human body contains a greater number of bacterial cells than any other cell type. Most of these cells are found in the gastrointestinal tract, particularly in the colon, with up to 10^{11} bacteria per gram of luminal contents residing there[146]. With such a large bacterial population, this community is the most metabolically active collection of

cells in the body[147]. Following the seminal observations by Backhed et al.[148] in 2004 linking the gut microbiota to obesity, the gut microbiota has now been linked to a whole host of diseases ranging from gastrointestinal disorders to type 2 diabetes.

It is becoming increasingly clear that early life factors influence the establishment of the infant microbiota and can have lasting effects on health throughout life[149]. Maternal microbiota composition has a large impact on that of the infant and can influence colonization as early as *in utero* through trans-placental transfer of maternal blood factors to the fetus, fetal ingestion of amniotic fluid, peri-natal colonization by maternal microbiota and maternal milk factors[33]. Bacteria have been repeatedly found in milk and have been shown to move from the intestinal mucosa to colonize other mucosal surfaces in the body including the lactating mammary gland, although the colonization is somewhat selective and regulated by lactogenic hormones[150]. Health status during pregnancy can also play a role in microbial colonization of the infant, as differences have been observed based on weight status and weight gain during pregnancy. Offspring of overweight women, or women who gained excess weight during pregnancy had lower concentrations of bifidobacteria[151]. Treatment with antibiotics can also impact early offspring microbial communities, with an increase in Enterobacteriaceae which lasted longer in pups than in the dams themselves. This profile, characteristic of inflammation, can lead to increased fat deposition[152].

Delivery method, be it vaginal or caesarean, can influence the establishment of different bacterial communities in the gut. With caesarean delivery, the direct contact with vaginal microbiota is absent, giving environmental and pathogenic bacteria greater opportunity for colonization[153]. Maternal microbiota during the first trimester has been shown to be most similar to that of their offspring at 4 years of age[26]. This early-pregnancy profile appears advantageous given that as pregnancy progresses there are fewer butyrate producers, less phylogenetic diversity and more Enterobacteriaceae, which are associated with inflammatory conditions[26]. There appears to be some discrepancy as to the importance of vaginal delivery and the *in utero* transfer of bacteria with the beneficial profiles observed in infants and the similarity to maternal microbiota during trimester 1 at 4 years of age in the child. It has been proposed that near the end of term,

changes in maternal immunity may increase bacterial translocation and the thinning of the placental barrier would increase exchange between mother and fetus[154, 155]. As the maternal microbiota has increasing numbers of Enterobacteriaceae throughout pregnancy it would be expected that increased circulation of bacteria would increase infant exposure and potential colonization by this negatively associated species, though this does not appear to be the case[26].

Formula-fed versus breastfed infants also have different microbiota profiles, with more bifidobacteria present in the feces of breastfed infants while formula-fed infants have a greater diversity of bacterial species[156]. Maternal diet can impact the microbiota and the gut development of the offspring[152]. Differences in microbiota have been observed between dams fed a regular diet and one supplemented with fructooligosaccharide[23] and when dams were fed a high-fat diet, where *Lactobacillus/Enterococcus* groups increased and *Bacteroides/Prevotella* groups decreased[157]. Prebiotics used only during pregnancy, and not into the suckling period, do not show transfer to the infant, despite changing maternal bacterial communities[158]. Maternal use of probiotics has also shown evidence for infant colonization, though findings have been inconsistent[156, 159]. Particularly during lactation the mother has a profound influence on offspring microbiota and this period appears to have greater impact than pregnancy[32]. Whether these changes or communities will persist into adulthood has not been examined, nor how they respond or recover after a high fat dietary challenge, which is known to be detrimental to the gut microbiota.

Diet continues to impact the gut microbiota throughout life, and changes may be positive or negative. Usually changes in response to diet will not persist beyond a couple of weeks and up to 5 weeks at most[27, 160, 161]. Changes in the composition of the gut microbiota have been observed in response to changes in any of the three macronutrients, either in proportion or type. A diet high in protein, with fat and fiber held constant, showed a decrease in *Bifidobacterium adolescentis* and increase in *Bacteroides B. fragilis* ss. *vulgatus,* although high variability throughout the study led the authors to express doubts as to the reliability of the results[30]. Diets with increasing proportions of protein and decreasing proportions of carbohydrate have been found to decrease total bacterial

numbers and the proportion of *Bacteroides* spp., *Bifidobacterium* spp. and *Roseburia-E. rectale;* all important butyrate producers[31].

Carbohydrates are the main fuel for bacterial metabolism. Prebiotic fibers in particular have been shown to have beneficial effects on the gut microbiota, and the remainder of this review will focus on inulin and oligofructose (OFS). Inulin has been found to greatly increase bacterial densities by approximately 55%[147]. Prebiotic supplementation has been found to increase members of the Bacteroidetes phyla and decrease Firmicutes and *Roseburia* spp.[162, 163]. As per the prebiotic definition, it has also been widely reported that prebiotic fiber increases bifidobacteria counts in cecal and fecal matter[25, 27, 118, 163-166]. This bifidogenic effect has important consequences for whole body health as bifidobacteria can decrease intestinal endotoxin levels and improve mucosal barrier function[25]. Consumption of a 10% OFS diet has been shown to improve glucose tolerance, even when consumed with a high fat diet, and correlates with increased *Bifidobacterium* spp.[167]. Bifidobacteria also negatively correlate with body weight and visceral fat mass, and inflammation as measured by plasma IL-1α and IL-6 and adipose mRNA concentrations of IL-1, TNF-α and plasminogen activator inhibitor type 1[167]. Bifidobacteria also inhibit growth of bacteroides, clostridia and coliforms, possibly due to their pH-lowering effect, or secretion of a bacteriocin-type substance[27]. Prebiotics have also been found to restore levels of the beneficial mucus-dwelling bacteria *Akkermansia muciniphila* after high fat feeding[168]. While it has been established that after direct prebiotic feeding effects last to a maximum of five weeks[27, 160, 161], long-term effects of maternal prebiotic consumption on the offspring, particularly in response to an adverse diet in adulthood, has not been examined.

The link between gut microbiota and metabolic disease was strengthened by the demonstration that lipopolysaccharide (LPS), an endotoxin found in the cell walls of gram-negative bacteria, compromises gut permeability and triggers metabolic endotoxemia[25]. Elevated plasma levels of LPS combined with a high fat diet induce inflammation and metabolic disease[25]. The LPS co-receptor CD14 appears to be necessary for these metabolic changes to occur[167]. However LPS-linked effects, such as poor glucose handling, inflammation, weight and fat gain are modulated by

bifidobacteria[167]. In fact, bifidobacteria are negatively correlated with inflammation, adiposity and glucose intolerance[167]. Despite being a gram-negative bacteria, *Akkermansia muciniphila* has been shown to decrease gut permeability, possibly through interactions with the mucous layer of the intestine, highlighting the complex interactions of the microbiota, their actions, components and location with the cells of the gastrointestinal tract[168].

As opposed to the benefits to gut microbiota associated with prebiotic fiber, consumption of a high fat diet compromises microbial profiles and function. Consuming a high fat diet has been shown to decrease overall bacterial counts while increasing the proportion of Bacteroidales and Clostridiales regardless of whether rats were resistant or prone to becoming obese on the high fat diet[169]. Animals that did become obese had an increase in Enterobacteriaceae, likely related to increased inflammation[169]. Specifically, the *E. rectale-C. coccoides* group has been shown to be lower in high fat fed mice[167]. High fat feeding also decreases *Bifidobacterium* spp. and is associated with increased endotoxemia, poor glucose handling, increased weight and visceral fat mass, and markers of inflammation[167]. New research has shown high fat feeding also decreases *Akkermansia muciniphila* which is associated with compromised gut barrier function[168].

It has been shown, although not unanimously, that the proportions of Firmicutes and Bacteroidetes differ between lean and obese individuals, with greater proportions of Firmicutes found in the obese[31, 170-172], and this effect is often reversed with weight loss[173]. The microbiota associated with an obese phenotype has shown a capacity for increased energy harvest due to increased fermentation[174]. This relationship between increased energy harvest and the microbial community has been demonstrated in many situations, with normal weight mice receiving microbiota from *ob/ob* littermates, germ-free mice showing a dramatic increase in weight when colonized with microbiota from *ob/ob* mice and even when receiving microbiota from women in their third trimester of pregnancy[26, 148, 174].

Many of the positive or negative effects associated with the proportions of various bacterial species are linked to the short-chain fatty acids (SCFA) produced by bacteria as

a result of fermenting non-digestible carbohydrates in the distal gut. Up to 10% of dietary energy may be released by fermentation in the form of SCFA[175]. Acetate, propionate and butyrate are the main SCFAs found in the large intestine. It has been shown that butyrate production decreases with decreasing carbohydrate intake, although butyrate concentrations can be somewhat maintained with a weight loss diet high in protein with moderate carbohydrate content[28, 31].

2.10 Summary of Current Knowledge

It is clear that maternal diet during pregnancy and/or lactation has a strong, lasting effect on the offspring and this pre-natal environment can in turn interact positively or negatively with the dietary environment into adulthood. The interplay between pre-and postnatal environments can influence multiple organs and systems, resulting in altered risk for numerous chronic diseases that impact the immediate and future generations. Most research completed to this point has examined worst case scenarios using adverse diets that would put any individuals' health at risk. With the examination of the high protein diet there is a shift towards a model that could be consumed by individuals with a belief that they are doing something beneficial for their health and it is important to continue to examine this diet in the context of the reproductive years. It is also important that other potentially healthful diets are examined in this context and research is conducted to potentially prevent adverse outcomes due to diet during pregnancy and in early infancy and promote health at critical periods of development. The studies that make up this thesis aimed to examine the balance of positive and negative consequences of maternal diets high in protein and prebiotic fiber. Prebiotic fiber consumption by offspring of protein-restricted dams was also examined.

Chapter Three: A maternal high protein diet predisposes female offspring to increased fat mass in adulthood whereas a prebiotic fiber diet decreases fat mass in rats[1] [2]

3.1 Introduction

The World Health Organization projected that as of 2005 there were 20 million overweight children and 400 million obese adults worldwide, a number that is predicted to reach 700 million by 2015[176]. The rapid rise in obesity rates has been theorized to be at least partially linked to developmental programming or the ability of an insult occurring at a critical period in development to result in persistent effects on metabolism and health[2]. Compromised nutritional exposure of the foetus *in utero* has been implicated in programming later cardiovascular disease, hypertension and diabetes in offspring[2]. Detrimental metabolic programming has been shown to affect birth size and body weight of future generations, even up to twelve generations in rodents[1].

Glucose and lipid metabolism are responsible for energy use and storage by the body and its various systems. Many of the genes that regulate glucose and lipid metabolism are nutrient-responsive and can therefore be up- or down-regulated in response to changes in diet composition[112]. Both fiber and protein are important dietary components that yield nutrient-gene interactions in the body[177]. The effects of consuming excess amounts of these macronutrients in the context of pregnancy and developmental programming are incompletely understood.

Depending on the type of dietary fiber examined, diets high in fiber can enhance satiety and reduce food intake, reduce hypercholesterolaemia, improve type 2 diabetes management[178, 179] and even prevent preeclampsia[21]. The societal trend towards

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[1] A version of this chapter has been published. Hallam MC, and Reimer RA. *A maternal high-protein diet predisposes female offspring to increased fat mass in adulthood whereas a prebiotic fibre diet decreases fat mass in rats.* British Journal of Nutrition, 2013. April 8 Epub ahead of print.

[2] A portion of this work was presented in part at Developmental Origins of Health and Disease, Munich, Germany, April 2010. *Sex differences in offspring body weight and food intake after prebiotic fiber or high protein maternal diet*. Also at Obesity 2010. *Pre- and neo-natal exposure to maternal high protein and prebiotic fiber diets impact offspring growth and lipid metabolism.* Obesity *18(Suppl 2s):*S75.

consumption of highly processed and calorically dense foods has resulted in a decreased intake of dietary fiber[180]. We have shown that a high prebiotic fiber diet (~5%) is tolerated and effective in reducing body weight, fat mass and food intake in humans[19]. These same effects can be seen in rodents fed diets containing 10-20% prebiotic fiber[181-183]. Despite the known benefits of fiber, pregnant women are not meeting current recommendations for dietary fiber intake[184, 185].

There are also large variations in protein intake in pregnant women with some reports of intake as high as 350 g/day of protein in the third trimester[104]. High protein intake during pregnancy have been linked to low birth weight[108], increased blood pressure[109, 186] and increased cortisol levels[110]. It has been previously shown in animal studies that a maternal diet high in protein can lead to fetal growth retardation and subsequently trigger increased fat mass in adulthood[22].

We have previously shown that a high protein diet introduced at weaning predisposes rats to an obese phenotype when they are given a high energy diet in adulthood; whereas consumption of a high prebiotic fiber diet during growth may provide some protection[113]. Furthermore, when dams were fed the same high protein and high fiber diets during pregnancy and lactation, plasma glucose at 28d of age was lower in high fiber versus control and high protein offspring and glucagon-like peptide-1 (GLP-1), a potent insulin secretagogue and anorexigenic hormone was increased in high fiber offspring[181]. In brown adipose tissue, high protein offspring had increased resistin and interleukin-6 mRNA expression, two factors associated with inflammation and insulin resistance. Because changes in offspring were only measured from postnatal days 7 through 35, we do not know if these early changes persist into adulthood and to what extent they affect glucose control and adiposity into adulthood.

Our objective, therefore, was to determine the long term effects of maternal diets high in protein or prebiotic fiber content on offspring glucose control and adiposity in adulthood. Specifically, we examined body weight, fat mass and the expression of satiety hormones and genes related to glucose control and lipid storage in offspring from dams consuming a control, high fiber or high protein diet during pregnancy and lactation. Given evidence that the developmental programming effects of early nutrition can be

latent, we used an 8 week high fat, high sucrose diet challenge in adulthood to unmask the potential effects of early programming.

3.2 Methods and Materials

3.2.1 Ethical Approval

The University of Calgary Animal Care Committee approved the experimental protocol which was conducted in accordance with the Guide for the Care and Use of Laboratory Animals.

3.2.2 Animals and Diets

Thirty-seven virgin Wistar dams were obtained from Charles River (Montreal, QC, Canada) and housed in a temperature and humidity controlled facility with a 12-h light/dark cycle. After 1 week of acclimatization, animals were given one of three nutritionally complete experimental diets: high prebiotic fiber (HF) (21.6% wt/wt, 1:1 ratio of oligofructose and inulin; 13.73 kJ/g), high protein (HP) (40% wt/wt; 15.74 kJ/g), or control (C) (based on AIN-93G; 15.74 kJ/g) (Tables 3.1 and 3.2). All maternal diets were mixed in house using ingredients purchased from Dyets, Inc. (Bethlehem, PA, USA)[112]. Dams consumed the diets for one week prior to being bred with male Wistar rats in wire-bottomed cages. Following the identification of a copulation plug, dams were housed individually and continued to consume their assigned experimental diet (C, HF, or HP) until the pups were weaned. Dams were weighed weekly, and food intake was measured throughout week 2 of pregnancy.

3.2.3 Body Composition

Pups were weighed on the day after birth, and litters then culled to 10 pups with equal numbers of males and females where possible. Offspring were weighed weekly for the remainder of the study. Food intake was also measured for 5 consecutive days out of every 20 days by subtracting the weight of the cup and diet from the previous days'

weight. At weaning (3 weeks), 1 male and 1 female pup were randomly selected from each litter to continue in the study until 22 weeks of age. By selecting one male and one female from each litter we examined n=10 individual rats per sex that were not all from one litter, minimizing the effect of any single dam. Pups were weaned onto AIN-93G control diet[187]. Offspring were then switched to AIN-93M (15.07 kJ/g) for maintenance at 10 weeks of age. At 14.5 weeks of age, offspring were fed a high fat, high sucrose (HFS) diet (19.26 kJ/g)[182] for 8 weeks (Figure 3.1). The HFS diet composition can be found in Tables 3.1 and 3.2. One male and one female pup not selected for the study underwent a DXA scan (Hologic ODR 4500; Hologic Inc.) while lightly anaesthetised using isoflurane one week post-weaning. Hologic QDR software for small animals was used to determine lean and fat mass. A separate group of pups referred to as reference rats (n=10 male and n=10 female offspring from n=5 control diet dams) were weaned at 3 weeks of age onto control diet (AIN-93G) and continued to consume this diet (AIN-93M after 10 weeks of age) throughout the study (i.e. no maternal intervention and no exposure to HFS diet). This reference group, matched for age and sex to the intervention groups, provides a standard of normal growth in these rats.

3.2.4 Oral Glucose Tolerance Test and Tissue Sampling

At the end of the 8 weeks of HFS diet consumption, rats were fasted overnight and an oral glucose tolerance test performed (OGTT). Blood was sampled from the tip of the tail in conscious rats followed by an oral glucose gavage (2 g/kg). At 15, 30, 60, 90 and 120 min post-glucose gavage, additional blood was sampled from the tail and immediately analyzed using a blood glucose meter (Accu-Chek Blood Glucose Meter, Laval, QC). One day prior to study termination, rats underwent a DXA scan under light anaesthetic as described above. A second OGTT for satiety hormone analysis was performed at the time of terminal tissue collection. After an overnight fast, rats were anaesthetized with isoflurane and a fasting cardiac blood sample was taken. Rats were then given 50% dextrose (wt/vol) by oral gavage at a dose of 2 g/kg. At 15, 30, 60 and 90 min post-gavage, another cardiac blood sample was taken according to our previous work while rats were anaesthetized[188]. Blood was collected in tubes containing diprotinin-A

(0.034 mg/ml blood; MP Biomedicals, Irvine, CA); Sigma protease inhibitor (1 mg/ml blood; Sigma Aldrich, Oakville, ON, Canada) and Roche Pefabloc (1mg/ml of blood; Roche, Mississauga, ON, Canada) and then centrifuged at 1600×g for 12 min at 4 C. Plasma was stored at -80ºC until analysis. The OGTT was a terminal procedure and after the 90 min blood collection rats were killed via over-anaesthetisation and aortic cut. The liver, stomach, small intestine, cecum and colon were weighed, a sample snap frozen in liquid nitrogen and stored at -80ºC.

3.2.5 Plasma Analysis

A Milliplex Rat Gut Hormone kit (Millipore, St. Charles, MO) and Luminex instrument were used to measure ghrelin (active), insulin, amylin (active), leptin, glucose-dependent insulinotropic polypeptide (GIP) (total) and peptide tyrosine tyrosine (PYY) (total). An ELISA was used to measure active GLP-1 (Millipore, St. Charles, MO). Non-esterified fatty acids (NEFA) at fasting were measured using an enzymatic colorimetric assay according to manufacturer instructions (Wako Diagnostics, Richmond, VA). Homeostasis model assessment of insulin resistance (HOMA-IR) was calculated from fasting insulin and fasting glucose.

3.2.6 Hepatic Triglyceride Analysis

Triglyceride content of the liver was quantified using 25mg of tissue according to the manufacturer guidelines of the GPO reagent set (Pointe Scientific Inc., Lincoln Park, MI).

3.2.7 RNA Extraction and Real-Time PCR

Total RNA was extracted from the stomach, small intestine, colon and liver using TRIzol reagent (Invitrogen, Carlsbad, USA). Reverse transcription was performed with an input of 1 μg of total RNA using the 1st strand cDNA synthesis kit for RT-PCR (Invitrogen, Carlsbad, CA USA) with oligo d(T)15 as a primer. The cDNA was amplified

using primers synthesized by the University of Calgary Core DNA Services (Calgary, AB, Canada) and analyzed by real time PCR. Primer sequences were according to our previous work[112]. A melt curve showed the melting point of the PCR product of interest. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was verified as a suitable housekeeping gene for the tissues of interest and GAPDH primers included as an internal control in the reactions. The $2^{-\Delta CT}$ method [ΔCT = CT (gene of interest) – CT (reference gene)] was utilized for the data analysis where threshold cycle (CT) indicates the fractional cycle number at which the amount of amplified target reaches a fixed threshold[189]. The ΔCT is the difference in threshold cycles for the gene of interest and GAPDH.

3.2.8 Statistical Analysis

All data are presented as mean ± SEM. Data collected from the dams was analyzed with one-way ANOVA with Tukey's post hoc analysis. In offspring, a two-way ANOVA was used to compare the main effects of diet and sex, and their interaction. Only when a significant interaction effect was identified were all 6 groups compared using a one-way ANOVA with Tukey's post hoc analysis. The reference group data is provided as a comparator for rats that did not undergo any intervention (either maternal diet manipulation or offspring HFS diet consumption). Given that there was no intervention and they were for reference purposes alone, the reference group was not included in the statistical analysis. Given the numerous variables examined in the offspring a Bonferroni correction was applied such that only $P \leq 0.01$ was considered significant. Statistical analysis was performed using PASW v 17.0 software (Chicago, IL).

Table 3.1. Diet Compositions

Ingredient	Control (growth)	High Protein	High Fiber	Control (maintenance)	High Fat/Sucrose
Cornstarch (g/kg)	397.5	197.5	253.3	465.7	47.5
Casein (g/kg)	200.0	400.0	174.5	140.0	140.0
Dyetrose (g/kg)	132.0	132.0	115.8	155.0	-
Sucrose (g/kg)	100.0	100.0	87.7	100.0	512.5
Soybean Oil (g/kg)	70.0	70.0	61.4	40.0	100.0
Lard (g/kg)	-	-	-	-	100.0
Alphacel (g/kg)	50.0	50.0	43.9	50.0	50.0
AIN-93M Mineral Mix (g/kg)	35.0	35.0	30.7	35.0	35.0
AIN-93-VX Vitamin Mix (g/kg)	10.0	10.0	8.8	10.0	10.0
L-Cystine (g/kg)	3.0	3.0	2.6	1.8	2.0
Choline bitartrate (g/kg)	2.5	2.5	2.2	2.5	3.0
Inulin (g/kg)	-	-	109.6	-	-
Oligofructose (g/kg)	-	-	109.6	-	-

Table 3.2. Macronutrient Compositions

% of Total Calories	Control (growth)	High Protein	High Fiber	Control (maintenance)	High Fat/Sucrose
Carbohydrate	63.80	44.67	64.16	73.98	49.75
Protein	19.45	38.59	19.26	14.11	11.13
Fat	16.74	16.74	16.58	9.99	39.12

Figure 3.1. Experimental design

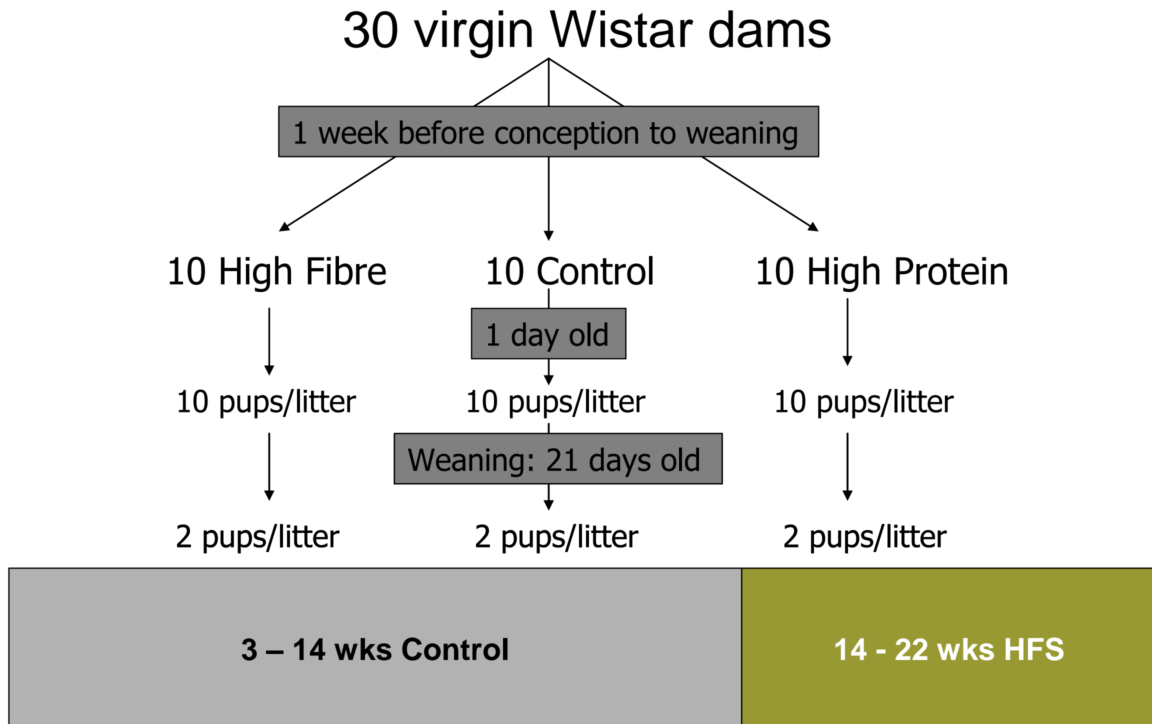

Figure 3.1: Schematic of experimental design.

3.3 Results

3.3.1 Dams and Litters

Of the 37 dams originally obtained for this study, 24 delivered viable litters. Of the control dams, 3 had spontaneous abortions, and one exhibited abnormal behaviour towards her litter. Abnormal behaviour consisted of splitting the nest of pups into two at opposite ends of the cage and tending to one nest more than the other, thus resulting in greater weight gain in pups of one nest over the other. Of the HP dams, 2 had a number of pups die within a week of delivery, resulting in litters too small to be used in this study. One HP dam delivered all still-born pups, and another also exhibited abnormal behaviour towards her litter. One HF dam did not conceive, one died during the first week of pregnancy from cardiac arrest, one became moribund after delivery of her pups, and another 2 died at one and two weeks, respectively, after delivery due to intestinal complications.

Total weight gain during pregnancy was greater in C and HP dams compared to HF ($P<0.05$, Table 3.3). The birth weight of female offspring from HF dams was lower than HP and C (Table 3.3) whereas male birth weights did not differ (hereafter offspring are referred to as HF1, HP1 or C1). There were no differences in number of pups delivered, number of males and females, or stillborns across the diet groups.

Table 3.3. Weight gain and litter statistics of dams fed a control, high protein or high prebiotic fiber diet during pregnancy and lactation

	Control	High Protein	High Fiber
Dam weight gain during pregnancy (g)	148.9±8.9^{a}	146.5±7.3^{a}	113.8±8.4^{b}
Dam energy intake (kJ/d)	432.2±23.8^{a}	468.2±20.9^{a}	344.3±20.9^{b}
Dam food intake (g/d)	27.5±1.5^{ab}	29.7±1.5^{a}	24.6±1.0^{b}
Female pup birth weight (g)	5.9±0.8^{a}	6.1±0.7^{a}	5.3±0.7^{b}
Male pup birth weight (g)	6.0±0.9	6.1±0.8	6.0±0.8
# Pups	14.8±1.0	15.1±0.8	14.8±1.0
# Males	7.8±0.8	8.4±0.7	7.0±0.8
# Females	7.0±0.8	6.5±0.6	7.8±0.7

Values are mean ± SEM with n=8-10 per group. The superscripts ^{a,b} are used to depict differences between diets wherein treatments without a common letter are different (*P*<0.05). Food and energy intake was measured during the second week of pregnancy.

3.3.2 Offspring Growth and Food Intake

As measured by DXA at 4 weeks of age, diet but not sex affected percent body fat with HF1 lower than C1 (C1: 20.0%±0.9, HP1: 18.4%±1.7, HF1: 15.4%±1.0; P=0.01). As expected with growth, there was a significant effect of week (P=0.0001) and week × sex (P=0.001) for body weight from 4 to 22 weeks of age (Figure 3.2) with males having higher body weight than females (Figure 3.2). Final body weight was affected by the interaction of diet and sex (P=0.01) wherein female HP1 had higher body weight than C1 and HF1 (Figure 3.3A). Similarly, for percent body fat, the interaction between diet and sex affected body fat (P=0.01) wherein female HP1 had higher body fat than C1 which in turn was higher than HF1 (Figure 3.3B). When maternal weight gain was examined as a covariate, no significant effect was found for female offspring final body weight (P=0.112) or percent body fat (P=0.069). There were no differences in naso-anal length within male or female offspring, and there were no differences between diet groups for any organ weights or lengths (data not shown). Independently, time and sex affected offspring's intake of the control diet from 4 to 13 weeks of age wherein food intake increased with increasing age and males consumed more food than females (P <0.01). There were no differences due to diet group for this period. When the rats were switched to the HFS diet, there was a significant effect of sex (P=0.001) wherein male rats consumed more of the diet than females (Figure 3.3C).

Figure 3.2. Offspring longitudinal body weight

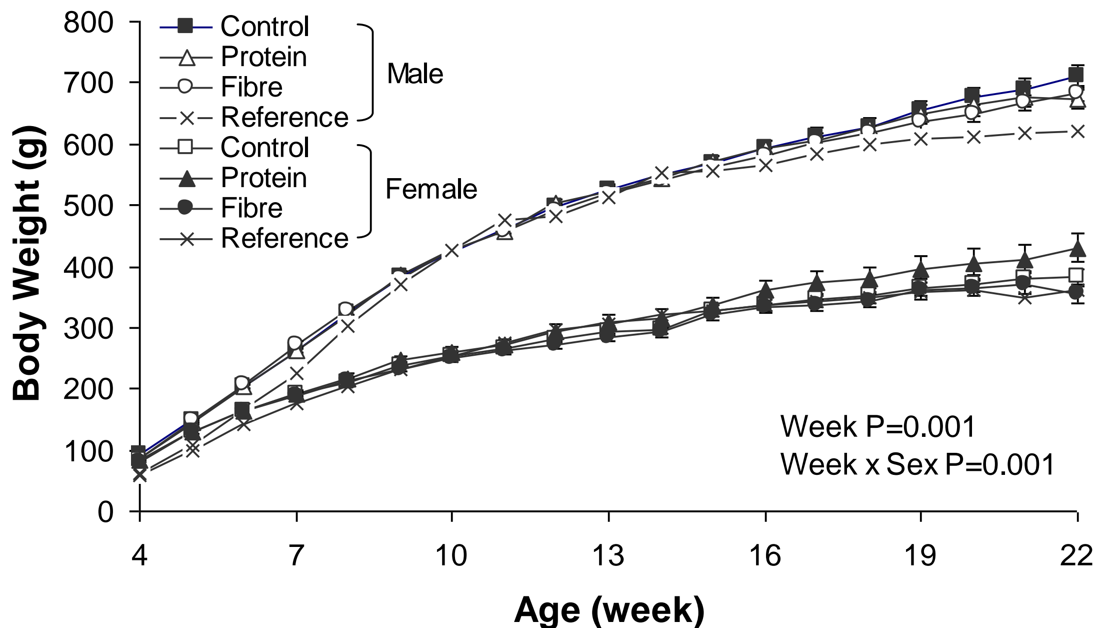

Figure 3.2: Longitudinal body weight in female and male offspring of dams fed a control, high protein or high prebiotic fiber diet during pregnancy and lactation. Results are mean ± SEM, n=10 M and n=10 F.

Figure 3.3. Final body weight and percent body fat of offspring at 22 weeks and average HFS diet intake

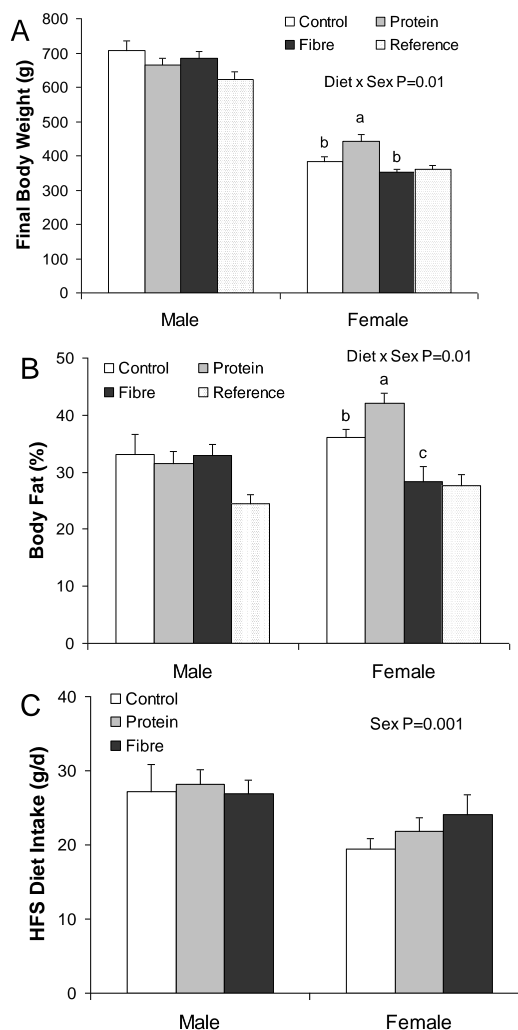

Figure 3.3: Final body weight, percent fat and high fat, sucrose diet intake in offspring of dams fed a control, high protein or high prebiotic fiber diet. Body weight (A) was greater in female HP1 than HF1 and C1 (P=0.043), percent fat (B) was greater in HP1 females than HF1 and C1, and high fat/sucrose diet consumption (C) was not different between diet groups for males or females. Results are mean ± SEM, n=10 M and n=10 F. The superscripts ^{a,b,c} are used to depict significant diet × sex differences wherein treatments without a common letter are different (P≤0.01).

3.3.3 Plasma Glucose and Satiety Hormones

Fasting values and AUC were examined for 7 appetite-regulating hormones. At fasting, there was a significant effect of sex on insulin (*P*=0.001) wherein males had higher levels than females (Table 3.4). In contrast, females had higher fasting levels of ghrelin (*P*=0.001) and GIP (*P*=0.002) than males. Only fasting GIP was significantly affected by diet (*P*=0.004) wherein HF1 was higher than C1 and HP1. For AUC, which represents the exposure to the hormone of interest over the entire course of the OGTT, females had higher ghrelin than males (*P*=0.001; Table 3.5). Diet affected GIP AUC (*P*=0.0013) with HF1 higher than HP1 but not C1. Similarly, PYY AUC was higher with HF1 (P=0.0015) compared to HP1 but not C1. There were no differences between sexes for GIP or PYY AUC. There were no differences in fasting or AUC for glucose (Tables 3.4 and 3.5). Independently, diet (*P*=0.004) and sex (*P*=0.001) but not their interaction affected HOMA-IR wherein males had higher values than females and C1 was higher than HP1 at the end of the study (Table 3.4).

Table 3.4. Fasting blood glucose and plasma satiety hormones in offspring

	Sex	Control	Protein	Fiber	Reference	Diet	Sex	D × S
Glucose*	M	5.2±0.2	5.4±0.2	5.9±0.4	4.6±0.2	0.216	0.134	0.547
(mmol/L)	F	6.0±0.4	5.5±0.3	6.2±0.5	5.4±0.5			
Insulin	M	3578±501	2703±790	3395±794	2385±425	0.428	0.001	0.576
(pg/mL)	F	2154±334	1680±326	1186±137	1147±175			
Amylin	M	50.4±3.6	46.0±4.6	58.5±7.4	77.9±7.8	0.476	0.079	0.049
(pg/mL)	F	65.6±4.6	59.0±4.1	51.0±3.9	80.0±8.4			
Ghrelin	M	187.2±25.8	185.6±18.4	267.2±33.0	133.4±24.5	0.046	0.001	0.735
(ng/mL)	F	410.3±28.0	376.9±17.9	446.0±47.5	348.3±77.9			
GIP (ng/mL)	M	37.8±4.7^{a}	32.4±3.0^{a}	58.6±6.9^{b}	51.0±4.2	0.004	0.002	0.255
	F	54.1±5.4^{a}	52.8±5.0^{a}	61.8±4.7^{b}	57.8±8.3			
PYY (pg/mL)	M	68.7±4.9	62.1±3.7	70.7±6.9	39.9±2.8	0.335	0.869	0.859
	F	68.1±3.3	64.0±4.4	67.6±3.4	57.8±5.1			
GLP-1	M	7.2±0.6	5.6±0.7	5.8±0.5	41.4±3.7	0.742	0.392	0.237
(pg/mL)	F	5.7±0.5	6.2±0.5	5.8±0.4	48.2±5.2			
Leptin	M	23.4±5.94	17.8±3.19	22.29±3.37	5.21±0.54	0.897	0.023	0.301
(ng/mL)	F	13.9±1.14	17.32±2.48	11.82±2.85	4.51±0.89			
HOMA-IR	M	18.8±1.8^{b}	9.5±2.0^{a}	13.7±2.3^{ab}	11.8±2.1	0.004	0.001	0.460
	F	11.0±2.0^{b}	6.4±2.2^{a}	6.3±2.3^{ab}	6.4±0.96			

Values are mean ± SEM with n=8-10 per group. Only P≤0.01 is considered significant. The superscripts ^{a,b} are used to depict differences between diets wherein treatments without a common letter are different.

*Glucose concentrations were measured during an OGTT done on conscious animals using a tail nick 1 week prior to termination while all other hormone data is from anaesthetised rats at the terminal OGTT. D, diet; S, sex.

Table 3.5. Area under the curve for blood glucose and plasma satiety hormones in offspring during the oral glucose tolerance tests

	Sex	Control	Protein	Fiber	Reference	Diet	Sex	D × S
Glucose*	M	998±27.6	931±30.8	977±41.5	955±90.0	0.12	0.49	0.47
(mmol/L/90 min)	F	1052±56.9	979±22.4	942±27.4	969±43.7			
Insulin (ng/mL/90	M	457±36.4	390±48.1	387±32.6	275±30.2	0.46	0.027	0.49
min)	F	330±41.8	366±58.3	296±38.6	202±18.3			
Amylin	M	5062±426	3865±264	5207±631	5785±587	0.023	0.71	0.016
(pg/mL/90 min)	F	5519±424	4598±295	3643±347	5765±669			
Ghrelin	M	13.0±2.6	12.1±1.8	17.2±2.3	14.6±1.9	0.41	0.001	0.59
(ng/mL/90 min)	F	24.9±2.8	22.5±3.1	24.2±1.2	31.8±4.4			
GIP (pg/mL/90	M	6285±589[ab]	3943±272[a]	7819±1871[b]	5853±1071	0.013	0.55	0.90
min)	F	6662±599[ab]	5031±1552[a]	7928±1699[b]	4068±284			
PYY (pg/mL/90	M	7793±600[ab]	5962±519[a]	8123±825[b]	3840±153	0.015	0.10	0.29
min)	F	7360±560[ab]	5868±339[a]	7262±502[b]	5230±358			
GLP-1	M	621±39.8	542±50.2	546±58.1	414±36.7	0.43	0.11	0.53
(pg/mL/90min)	F	517±42.5	535±25.4	479±30.2	482±52.0			
Leptin	M	2398±590	1870±330	1807±209	563±78	0.35	0.039	0.24
(ng/mL/90min)	F	1297±160	1919±318	1047±239	405±51			

Values are mean ± SEM with n=8-10 per group. Only P≤0.01 is considered significant. The superscripts [a,b] are used to depict differences between diets wherein treatments without a common letter are different.

*Glucose data was taken from an OGTT done on conscious animals using a tail nick 1 week prior to termination while all other hormone data is from anaesthetised rats at the terminal OGTT. D, diet; S, sex

3.3.4 NEFA and Liver Triglycerides

Independently, maternal diet (*P*=0.001) and sex (*P*=0.001) affected liver triglyceride content. Hepatic triglyceride concentration was lower in females than males and lower with HF1 than C1 or HP1 (Figure 3.4A). Independently, maternal diet (*P*=0.001) and sex (*P*=0.001) also affected plasma NEFA concentrations wherein males had higher levels than females and HP1 was higher than C1 and HF1 (Figure 3.4B).

3.3.5 Gastrointestinal Tract Gene Expression

In the stomach, maternal diet affected ghrelin gene expression (*P*=0.01) with HP1 having greater levels than HF1 (Table 3.6). The interaction of maternal diet and sex affected mRNA levels of SGLT1 (*P*=0.014) in the ileum wherein male HF1 had lower levels than male HP1 and female C1. Maternal diet alone affected expression of GLUT2 (*P*=0.002) wherein HP1 and HF1 were lower than C1.

3.3.6 Hepatic Gene Expression

Maternal diet affected hepatic FAS gene expression (*P*=0.006), with HP1 having lower levels than HF1 and C1 (Table 3.7). PEPCK (*P*=0.006) differed between sexes wherein males had higher mRNA levels than females. The interaction between maternal diet and sex affected PGC1α (*P*=0.006). PGC1α mRNA levels were lower in all males and HF1 females compared to female C1. There were no differences in SREBP1c or ACC1 expression (data not shown).

Figure 1.4. Offspring liver triglyceride and fasting plasma NEFA measures at 22 weeks

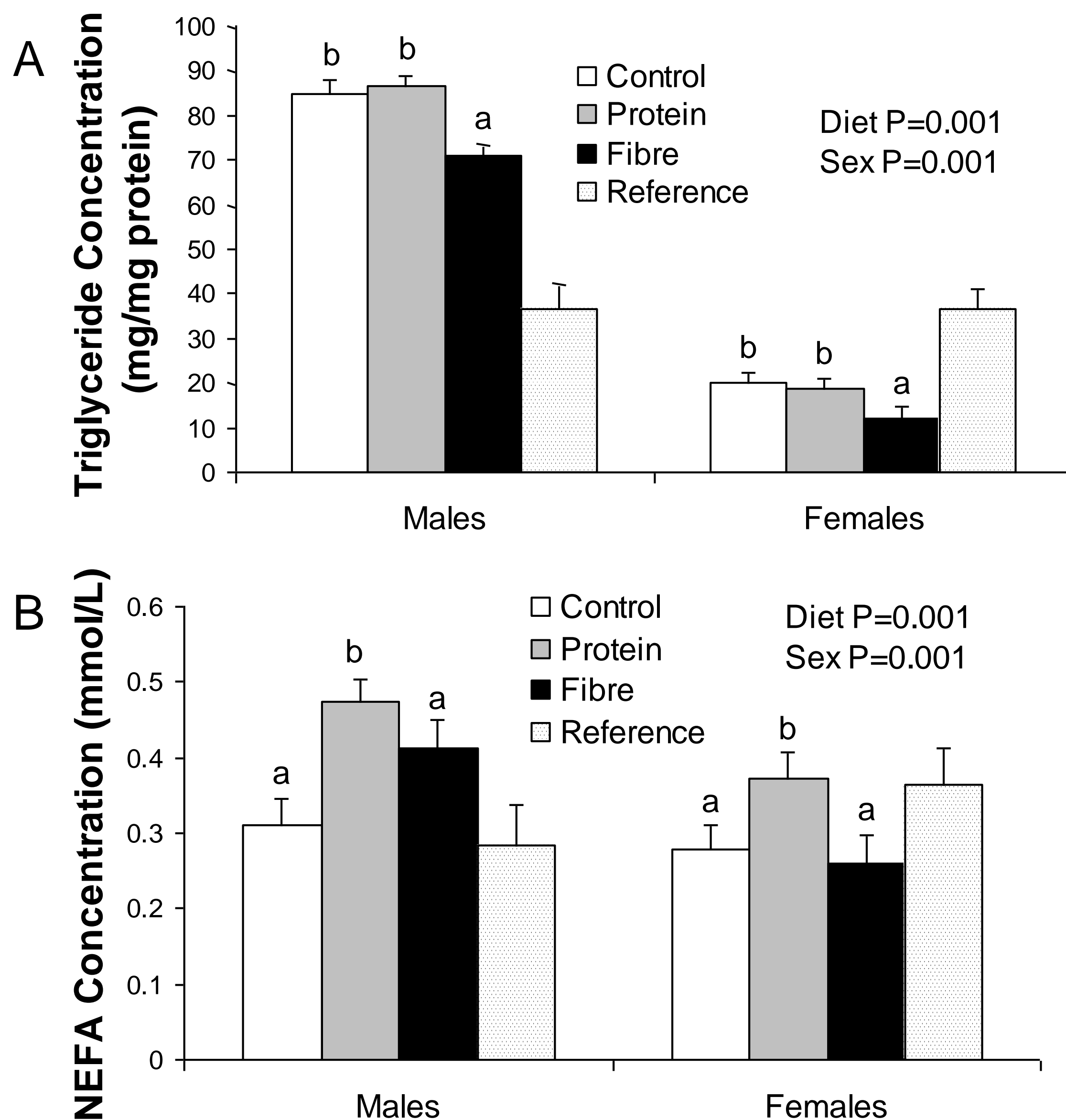

Figure 3.4: Hepatic triglyceride content (A) and plasma non-esterified fatty acids (B) in female and male offspring of dams fed a control, high protein or high prebiotic fiber diet during pregnancy and lactation. Results are mean ± SEM, n=10 M and n=10 F. The superscripts [a,b] are used to depict differences between diets wherein treatments without a common letter are different ($P \leq 0.01$).

Table 3.6. Intestinal gene expression in offspring of dams fed a control, high protein or high prebiotic fiber diet during pregnancy and lactation

	Sex	Maternal Diet			2-way ANOVA P-values		
		Control	High Protein	High Fiber	Diet	Sex	D × S
Stomach:	M	71.1±27.0^{ab}	93.3±32.0^{b}	46.4±15.5^{a}	0.010	0.12	0.15
Ghrelin	F	89.3±29.0^{ab}	205.5±61.7^{b}	30.6±11.8^{a}			
Duodenum:	M	22.1±1.8	27.3±3.8	26.0±3.2	0.029	0.458	0.089
GIP	F	22.3±2.3	31.4±4.4	15.8±2.4			
Ileum:	M	46.2±12.1	64.5±13.9	96.1±19.3	0.018	0.019	0.035
Proglucagon	F	60.9±8.8	194.6±52.7	104±20.3			
SGLT1	M	43.4±4.6^{xy}	55.6±11.5^{y}	26.4±2.9^{x}	0.012	0.84	0.014
	F	58.0±7.6^{y}	33.0±3.3^{xy}	37.7±5.3^{xy}			
GLUT2	M	22.5±6.9^{b}	6.9±1.7^{a}	14.1±4.9^{a}	0.002	0.18	0.65
	F	19.6±5.2^{b}	4.9±1.1^{a}	4.7±1.3^{a}			
Colon:	M	33.3±8.5	12.7±3.8	11.5±4.3	0.040	0.039	0.52
PYY	F	53.2±11.5	18.6±5.1	41.7±21.6			

Values are mean ± SEM with n=8-10 per group. Only P≤0.01 is considered significant. The superscripts ^{a,b} are used to depict differences between diets wherein treatments without a common letter are different. The superscripts ^{x,y} are used to depict significant diet × sex differences wherein treatments without a common letter are different. D, diet; S, sex.

Table 3.7: Hepatic gene expression in offspring of dams fed a control, high protein or high prebiotic fiber diet during pregnancy and lactation

	Sex	Maternal Diet			2-Way ANOVA P Values		
		Control	High Protein	High Fiber	Diet	Sex	D × S
FAS	M	1.4±0.3^{b}	0.86±0.24^{a}	1.8±0.5^{b}	0.006	0.18	0.31
	F	1.7±0.4^{b}	0.75±0.11^{a}	3.1±1.0^{b}			
PEPCK	M	4.3±0.8	3.9±0.7	7.5±1.2	0.11	0.006	0.037
	F	2.8±0.8	3.9±0.7	3.0±0.7			
PGC-1α	M	2.6±0.9^{x}	1.8±0.4^{x}	2.7±0.7^{x}	0.004	0.001	0.006
	F	8.1±0.9^{y}	4.7±1.2^{xy}	2.9±0.9^{x}			

Values are mean ± SEM with n=8-10 per group. Only P≤0.01 is considered significant. The superscripts ^{a,b} are used to depict differences between diets wherein treatments without a common letter are different. The superscripts ^{x,y} are used to depict significant diet × sex differences wherein treatments without a common letter are different.
D, diet; S, sex.

3.4 Discussion

Maternal diet is a well-recognized environmental factor that influences the health of offspring later in life and is in part attributed to epigenetic changes[190]. In our work examining the influence of maternal diets high in protein or prebiotic fiber, we show a difference in susceptibility to an obese phenotype in the offspring of dams fed these diets and a distinct sex effect; females being affected while males were not. The pattern of growth and development in these offspring may shed some light on the lasting influence of maternal diet. Accelerated postnatal growth or 'catch-up' growth has been associated with later metabolic disease and susceptibility to obesity[191]. We observed catch-up growth in HF1 offspring during the first 2 weeks of life. This rapid growth was evident in the female HF offspring wherein their birth weight was significantly lower than the HP1 and C1 female pups but no longer different from the other groups at weaning. This observation is interesting on several fronts given that there were no lasting detrimental effects on adiposity and the availability of food to the dams did not change, although offspring may have altered their suckling habits or maternal diet may have affected milk composition. While it has recently been shown that milk from HP dams compromises offspring growth due to impaired lactational function[137], it is not known how the HF diet affects milk composition. Maternal weight loss during lactation does not appear to explain the differences in rate of growth in the pups given that there were no differences among the control, HP and HF dams. Maternal weight gain during pregnancy, however, was affected by diet and HF dams gained less weight compared to HP and C dams. It may be tempting to speculate that weight gain, independent of dietary exposure, could have influenced offspring growth, however, we have recently demonstrated that this may not be the case. Consistent with the magnitude of differences in maternal weight gain during pregnancy achieved in this study, we recently showed that offspring body weight was not altered when maternal weight gain differed by 30-35 g on AIN-93G based diets (unpublished results). This is in contrast to a maternal HFS diet which not only resulted in higher pregnancy weight gain but likely interacts with the fat and sugar content of the diet to produce increased offspring body weight as well (unpublished results). Outside of maternal influences, it is possible that catch-up growth in the HF1 female offspring was

influenced by changes to gut microbial communities. Given the known bifidogenic effect of prebiotics, the HF1 offspring may have acquired a unique profile of bacterial species, or simply a greater number of bacteria that could have led to an initial increase in energy harvest similar to that which has been seen with germ free mice colonized with microbiota[174].

It is not clear what factors are responsible for the catch-up growth seen in the HF1 animals but it is clear that they avoided the predicted increase in body weight and adiposity typically seen with accelerated postnatal growth[192]. In fact at 4 weeks of age, the percent body fat was lower in HF1 males and females than C1. By the end of the study, male body weight and fat mass did not differ from the other two groups but HF1 females retained a lower body weight and percent body fat than HP1 offspring. Fat mass in the HF1 females was not only lower than HP1 but C1 as well. The elevated percent body fat in HP1 is consistent with other studies showing increased fat mass in offspring of dams fed a high protein diet during lactation[193]. Part of the reason behind this shift to fat mass may be impaired muscle growth which has been shown in offspring suckled by HP dams[106]. Although we did not observe the growth retardation in HP1 offspring that has been demonstrated previously[194], it is possible that growth restriction of lesser magnitude could have predisposed the HP1 animals to increased adipose accumulation as adults, especially when exposed to an HFS diet in adulthood.

Maternal satiety has been implicated as a key factor influencing catch-up weight gain, with levels of leptin and ghrelin in cord blood able to predict catch-up growth in humans[195, 196], which in turn relate to food intake later in life[197]. While plasma concentrations of these satiety hormones were not measured in our offspring at birth, we speculate that they may have played a role, especially since HF1 female offspring consumed ~25% more HFS diet from 14-22 weeks of age than the C1 females (although this did not reach statistical significance). In our previous work we observed a decrease in plasma GLP-1 and amylin at 7 days of age in offspring of dams fed HF versus HP diet[181]. Both GLP-1 and amylin reduce food intake and their lower levels at birth could be associated with greater food consumption and accelerated growth. Despite lower birth weight in females, early catch-up growth, and higher intake of HFS diet in adulthood,

HF1 offspring did not gain excessive body weight or fat, suggesting other mechanisms were at work that had greater influence than the catch-up growth itself. While glucose control was not negatively impacted in HF1 compared to controls, HOMA-IR scores were still higher than HP1. It is possible that the catch-up growth had a negative impact on insulin sensitivity that may not be fully apparent until an older age. Other negative effects of catch-up growth, such as decreased longevity and/or senescence in various tissues[197] may also develop but were not measured in the current study, and may have been counteracted by exposure to prebiotic fiber, which has been shown to increase longevity[198].

Offspring of HF dams had lower levels of liver triglycerides than HP1. While elevated plasma triglycerides has been shown in offspring of dams fed a high protein diet during lactation[193], the triglyceride lowering effect of the HF diet is a novel finding. Oligofructose has been shown to decrease the production of triglycerides in the liver, as well as increase catabolism of lipoproteins rich in triglycerides[199]. In our study, the reduced hepatic triglyceride content is intriguing given that the rats were not directly exposed to oligofructose, nor were there diet differences in the expression of acetylCoA carboxylase, an enzyme in the fatty acid synthesis pathway. NEFA levels were also elevated in our HP1 animals. This could be a reflection of the programmed preference for oxidative metabolism that has been shown by Rehfeldt et al.[106] in animals suckled by HP dams. The differences in body weight in females could be partially due to the decreased availability of NEFA, which may be involved in the regulation of hepatic fatty acid metabolism although despite similar decreases in NEFA in males they did not have lower body weight[200]. The decreased expression of FAS in our HP1 animals may be affected by increased availability of NEFA, impacting hepatic fatty acid metabolism. A similar decrease in FAS mRNA was observed in our previous work in rats aged 28 and 35 days[113]. This may be a lasting effect from the maternal diet, as diets low in carbohydrates and high in protein have been shown to decrease hepatic FAS mRNA[201-204]. Male HP1 animals showed decreased PGC-1α expression, which is characteristic for animals consuming a high fat diet and/or with hypertriglyceridemia[205]. This

decreased expression, along with decreased PEPCK expression could be related to their improved HOMA-IR score.

Decreases in the expression of sodium and glucose transporters, GLUT2 and SGLT-1, in the ileum of HF1 and HP1, and HF1 males, respectively, could be linked, particularly in HP1, to improvements in glucose homeostasis as demonstrated by improved HOMA-IR scores. However, SGLT1 mRNA expression in particular, is not always related to SGLT1 activity, as protein levels are more influential than mRNA levels[206]. Decreases in the expression of these transporters in HF1 may be related to differences in the gut microbiota, which remains to be examined. Should there be an alteration in the microbial community leading to increased fermentation, and therefore increased short-chain fatty acid production, the decreased carbohydrate content in the lumen would result in decreased expression of SGLT1. Glucose transport has been shown to decrease in the distal portions of the small intestine, and the decrease is more dramatic as rats age[207]. This could contribute to the differences seen in SGLT1 expression in the ileum.

We have previously demonstrated that increases in GLP-1 and PYY secretion along with upregulation of PYY and proglucagon expression occur in response to prebiotic consumption in rats[113, 188]. In this model only the dams consumed the prebiotic fiber and therefore we might expect that the effect may not be passed on to the offspring. This is largely confirmed although the HF1 offspring did have higher PYY AUC than HP1 offspring but not C1. We acknowledge that the use of anesthesia during the OGTT could potentially influence the concentrations of satiety hormones in the plasma, although Zardooz et al.[208] showed that isoflurane had no effect on glucose and insulin levels in fed rats and decreased insulin but not glucose in fasted rats. Similarly, Andrikopoulos et al.[209] showed that there was no difference in blood glucose concentrations and the ability to differentiate glucose tolerance in chow-fed versus high fat diet fed mice under anesthesia or conscious was the same. While the plasma concentrations of satiety hormones obtained in our anesthetized rats are within expected ranges, we have recently made procedural advancements that allow us to obtain sufficient blood from the tip of the tail in conscious rats and thereby avoid the use of anesthesia.

While there were no differences in male body weight or composition, males did exhibit changes in hepatic and intestinal gene expression, as well as differences in satiety hormones and hepatic lipid storage and glucose control as measured by liver triglycerides, plasma NEFA, and HOMA-IR. It is possible that prolonged exposure to the HFS diet could eventually result in differences in adiposity in response to the observed changes related to hepatic lipid storage and metabolism, especially since significant differences in female body weight were not apparent until 22 weeks. Sex differences observed here, wherein females were more affected than males, could be due to differences in placental gene expression. It has been shown that placentae of females have been found to have twice as many changes in gene expression compared to the placentae of males, making females adapt much more to environmental changes, such as diet[210].

In conclusion, we demonstrate that *in utero* exposure to a diet high in protein or prebiotic fiber has a lasting effect on offspring adiposity, hepatic lipid storage and expression of genes related to glucose and lipid metabolism. Within the time frame we examined, the effect was more pronounced in female than male offspring. Taken together these findings suggest that a maternal diet high in protein appears to have some adverse effects, particularly in regards to body composition, while a high prebiotic maternal diet appears to provide some protection against an obese phenotype in offspring once they reach adulthood.

Chapter Four: Maternal diets high in protein or prebiotic fibre differentially affect maternal milk composition and gut microbiota in rat dams and their offspring[3] [4]

4.1 Introduction

A growing body of evidence links the microbial community in the gut to regulation of body weight and metabolic health[24]. The gut microbiota functions to carry out processes that the human body would be otherwise unable to do, such as degrade and ferment certain carbohydrates, synthesize vitamins, and to increase energy and nutrient harvest[211]. By fermenting dietary components that humans and animals are normally unable to utilize, gut microbiota supply short chain fatty acids (SCFAs) that in themselves have been shown to have numerous health-promoting properties[212].

Free oligosaccharides are fermentable biomolecules that are present in all mammals' milk and provide benefits beyond essential nutrients. Numerous studies on human milk have concluded that the oligosaccharides, bacteria and other components in milk perform important biological functions including the establishment of intestinal microbiota and prevention of pathogen binding to intestinal cells, and have direct effects on the immune system[213-215]. Dietary intake during the neo-natal period, chiefly provided in humans as maternal milk or bovine milk-based infant formula, has a remarkable influence on the gut microbiota. Breastfed infants display a very high proportion of bifidobacteria which is in contrast to the increased microbial diversity and lower bifidobacteria detected in infants fed standard infant formula[156, 216]. Supplementing maternal diet with fructooligosaccharides, a prebiotic with bifidogenic properties, has also been shown to alter gut microbiota in dams and pups in mice[23]. Furthermore, evidence suggests that there is a similarity in the cecal microbiota of

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[3] A version of this paper was submitted to PLoS One and was favourably reviewed by 2 of the 3 reviewers. The third reviewer had concerns that prevented the manuscript from being accepted. The suggested revisions have been made and the manuscript has now been submitted to The Journal of Physiology.
[4] A portion of this work was presented in part at the Canadian Obesity Network Summit, April 2011. *Consumption of high prebiotic ifber or high protein diet during pregnancy alters gut microbiota in the dams and their offspring.* Canadian Journal of Diabetes, *35*(2): 205.

offspring and their mothers, between offspring of different litters with the same mother, and even between cousins[217], although it appears suckling has the greatest impact on similarity[32].

Two common prebiotics used to stimulate the growth of beneficial gut microbiota are inulin and oligofructose[27]. Inulin is a prebiotic derived from the chicory root and has been shown to increase bifidobacteria and lactate[164, 218]. Oligofructose, a shorter chain fructan derived from inulin, has also been shown to increase bifidobacteria, lactobacilli, and the *Clostridium coccoides-Eubacterium rectale* cluster[163, 219]. It also increases the insulinotropic hormone, glucagon-like peptide-1 (GLP-1)[220] and the related peptide GLP-2[219]. GLP-2 is a gut trophic factor and along with changes in the tight junction proteins zonula occludens (ZO-1) and occludin, oligofructose intake is able to improve gut barrier function and reduce plasma lipopolysaccharide (LPS), a known endotoxin and inflammatory agent[167].

Although previous studies have demonstrated that a maternal high protein diet[221] and a postnatal weaning diet high in protein [112] increase susceptibility to obesity in rats, investigations into the effects of these diets on the gut microbiota are limited but nevertheless consistent in their findings. Increased protein, whether through increased beef consumption or a protein supplement, decreases total numbers of bacteria[28, 31], numbers of *Bifidobacterium* spp.[29, 30], and proportions of *Roseburia/E. rectale* within the *Firmicutes* phylum and members of *C. coccoides*[28, 29, 31]. In dams fed a high protein diet during lactation, milk lactose content and milk yield is reduced compared to control. This compositional change has been linked to a slower rate of growth for pups during the lactation period[137]. The extent of the effects of a maternal high protein diet on offspring gut microbiota composition and development is not known, particularly the lasting effects into adulthood.

Given the interdependent relationship of offspring gut microbiota with maternal microbiota, our objective was to examine the effect of maternal diets high in protein or prebiotic fiber on offspring gut microbiota. We examined the gut microbiota present in dams at parturition, and compared them with that of the dams two weeks post-partum and that of their offspring two weeks after weaning and in early adulthood after a high fat

high sucrose dietary challenge. We hypothesized that microbiota profiles would be similar over time in the dams and would be predictive of the microbial profiles of the offspring. We also examined the composition of maternal milk, including oligosaccharides, to determine if diet-related changes in key milk components could influence offspring microbiota.

4.2 Methods

4.2.1 Ethical Approval

The University of Calgary Animal Care Committee approved the experimental protocol (Protocol Number: BI10R-10) which was conducted in accordance with the Guide for the Care and Use of Laboratory Animals. Isoflurane was used as anaesthetic where appropriate and all efforts were made to minimize suffering.

4.2.2 Animals and Diets

Thirty-seven virgin Wistar rat dams were obtained from Charles River (Montreal, QC, Canada), and housed in a temperature and humidity controlled facility with a 12-hour light/dark cycle. After 7 days of acclimatization animals were given one of three nutritionally complete experimental diets: high prebiotic fiber (HF) (21.6% wt/wt, 1:1 ratio of oligofructose and inulin), high protein (HP) (40% casein wt/wt), or control (C) (based on AIN-93G) according to our previous work[221]. The detailed composition of the diets can be found in Tables 4.1 and 4.2[112]. Dams consumed the diets for one week prior to being bred with male Wistar rats in wire-bottomed cages. Following the identification of a copulation plug, dams were housed individually and continued to consume their assigned experimental diet until the pups were weaned. Dams were weighed weekly. A reference group of dams (n=5) who consumed the control diet throughout the study were also included.

Pups were weighed the day after birth, and litters were culled to 10 pups with equal numbers of males and females where possible, and were weighed weekly for the

remainder of the study. At weaning (21d), 1 male and 1 female pup were randomly selected from each litter to continue in the study and were weaned onto the control diet (AIN-93G). At 10 weeks of age the offspring were switched to maintenance formula of the diet (AIN-93M). At 14.5 weeks of age, offspring were fed a high fat, high sucrose (HFS) diet for 8 weeks as a metabolic challenge (Figure 4.1). Pups from the reference dams (n=10 male and n=10 female) were weaned at 3 weeks onto control diet (AIN-93) and continued to consume this diet throughout the study. The reference group, matched for age and sex to the intervention groups, provides a standard of normal growth in these rats. At study termination, rats were fasted overnight and then anaesthetized with isoflurane. A fasting cardiac blood sample was taken for analysis of LPS (Pyrogene Recombinant Factor C Assay, Lonza Group Ltd.) and an oral glucose tolerance test was performed (results previously reported [221]). At the end of the OGTT rats were killed via overanesthetization and aortic cut.

4.2.3 Fecal Collection

Fecal samples were collected from the dams at parturition and 2 weeks post-partum. Offspring fecal samples were collected from the offspring at 5 weeks and at the end of the study. Cecal digesta was also collected when rats were euthanized.

4.2.4 Gut Microbiota Profiling using qPCR

Microbial profiling was performed according to our previous work [183]. Briefly, total bacterial DNA was extracted from fecal/cecal samples using FastDNA Spin Kit for Feces (MP Biomedicals, Lachine, QC, Canada) and quantified using PicoGreen DNA quantification kit (Invitrogen, Carlsbad, CA, USA). All samples were brought to a concentration of 4ng/ul prior to storage at -20ºC for later analysis. Amplification and detection were conducted in 96-well plates with SYBR Green 2 x qPCR Master Mix (BioRad). Samples were run in duplicate with a final volume of 25 ul containing 0.3 uM primer and 20 ng template genomic DNA. Group specific primers are provided in Table 4.3. The specificity of the primers and the limit of detection were determined according

to Louie et al. [222]. The 16S rRNA gene copies value was calculated according to the following webpage: http://cels.uri.edu/gsc/cndna.html using average genome sizes. Standard curves were normalized to the copy number of the 16S rRNA gene obtained from the following webpage: http://rrndb.mmg.msu.edu/index.php.

Table 4.1. Diet Compositions

Ingredient	Control (growth)	High Protein	High Fiber	Control (maintenance)	High Fat/Sucrose
Cornstarch (g/kg)	397.5	197.5	253.3	465.7	47.5
Casein (g/kg)	200.0	400.0	174.5	140.0	140.0
Dyetrose (g/kg)	132.0	132.0	115.8	155.0	-
Sucrose (g/kg)	100.0	100.0	87.7	100.0	512.5
Soybean Oil (g/kg)	70.0	70.0	61.4	40.0	100.0
Lard (g/kg)	-	-	-	-	100.0
Alphacel (g/kg)	50.0	50.0	43.9	50.0	50.0
AIN-93M Mineral Mix (g/kg)	35.0	35.0	30.7	35.0	35.0
AIN-93-VX Vitamin Mix (g/kg)	10.0	10.0	8.8	10.0	10.0
L-Cystine (g/kg)	3.0	3.0	2.6	1.8	2.0
Choline bitartrate (g/kg)	2.5	2.5	2.2	2.5	3.0
Inulin (g/kg)	-	-	109.6	-	-
Oligofructose (g/kg)	-	-	109.6	-	-

Table 4.2. Macronutrient Compositions

% of Total Calories	Control (growth)	High Protein	High Fiber	Control (maintenance)	High Fat/Sucrose
Carbohydrate	63.80	44.67	64.16	73.98	49.75
Protein	19.45	38.59	19.26	14.11	11.13
Fat	16.74	16.74	16.58	9.99	39.12

Figure 4.1. Experimental design

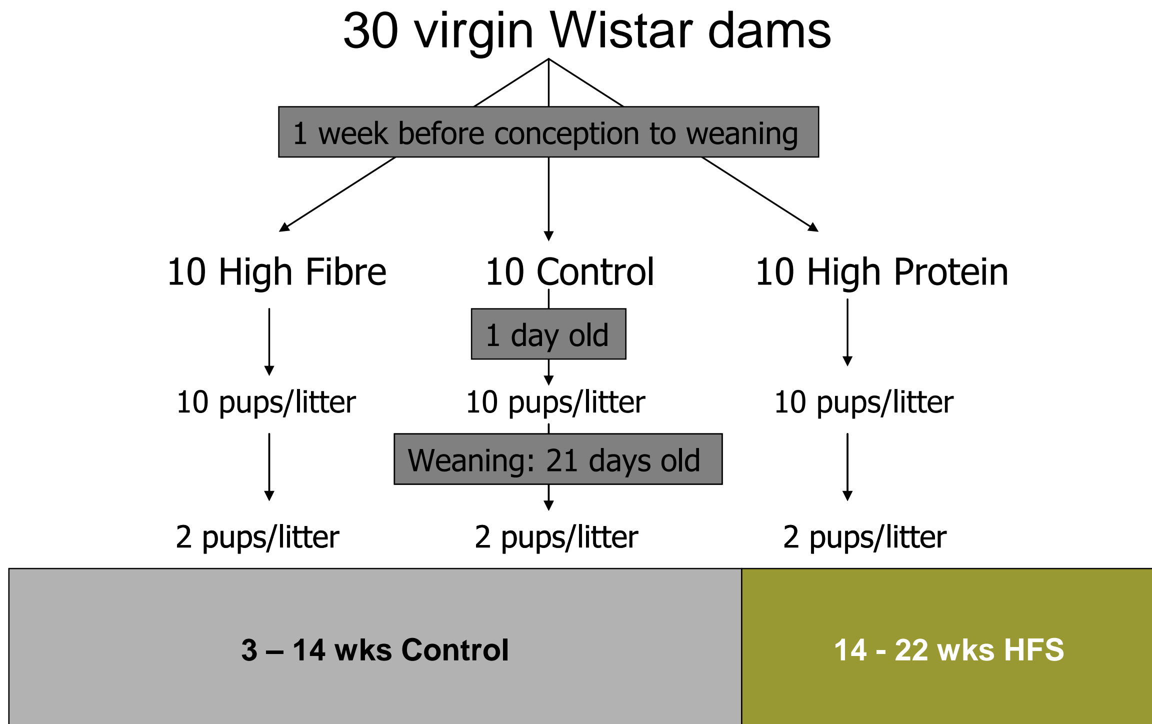

Figure 4.1: Schematic of experimental design.

Table 4.3. Gut microbiota primers and bacteria genomic DNA standards for qPCR

Group	Primers (F and R)	Genomic DNA Standard	Reference
Total bacteria	F: ACTCCTACGGGAGGCAGC R:GTATTACCGCGGCTGCTG	Escherichia coli	(Amann et al., 1990; W. T. Liu, Mirzabekov, & Stahl, 2001)
Firmicutes			
Clostridium leptum (cluster IV)	F: GCACAAGCAGTGGAGT R: CTTCCTCCGTTTGTCAA	Clostridium leptum	(Matsuki, Watanabe, Fujimoto, Takada, & Tanaka, 2004)
Clostridium coccoides (cluster XIV)	F: ACTCCTACGGGAGGCAGC R: GCTTCTTAGTCARGTACCG	Ruminococcus productus	(Amann et al., 1990; Franks et al., 1998)
Clostridium group (cluster I)	F: ATGCAAGTCGAGCGAKG R: TATGCGGTATTAATCTYCCTTT	Clostridium perfringens	(Rinttila, Kassinen, Malinen, Krogius, & Palva, 2004)
Clostridium group (cluster XI)	F: ACGCTACTTGAGGAGGA R: GAGCCGTAGCCTTTCACT	Clostridium difficile	(Song, Liu, & Finegold, 2004)
Lactobacillus	F: GAGGCAGCAGTAGGGAATCTTC R: GGCCAGTTACTACCTCTATCCTTCTTC	Lactobacillus jensonii	(Delroisse et al., 2008)
Roseburia	F : TACTGCATTGGAAACTGTCG R: CGGCACCGAAGAGCAAT	Roseburia hominis	(N. Larsen et al., 2010)
Bacteriodetes			
Bacteroides/ Prevotella	F: TCCTACGGGAGGCAGCAGT R: CAATCGGAGTTCTTCGTG	Bacteroides thetaiotaomicron	(Bernhard & Field, 2000; Nadkarni, Martin, Jacques, &

			Hunter, 2002)
Actinobacteria			
Bifidobacterium	F: CGCGTCYGGTGTGAAAG R: CCCCACATCCAGCATCCA	Bifidobacterium adolescentis	(Delroisse et al., 2008)
Archea			
Methanobrevibacter	F: CTCACCGTCAGAATCGTTCCAGTC R: ACTTGAGATCGGGAGAGGTTAGAGG	M. smithii	Beacon Designer 3.0
Proteobacteria			
Enterobacteriaceae	F: CATTGACGTTACCCGCAGAAGAAGC R: CTCTACGAGACTCAAGCTTGC	Escherichia coli	(Bartosch, Fite, Macfarlane, & McMurdo, 2004)

4.2.5 Milk Sample Collection and Fat and Protein Concentration

Milk was collected from twenty four of the dams at weaning (n=10 C; n=8 HP; n=6 HF). Dams were anaesthetized using isoflurane then given an intraperitoneal injection of oxytocin (2 IU). After 10 minutes, milk was collected using capillary tubes, one of which was spun using a hematocrit spinner (Iris Sample Processing StatSpin CritSpin Westwood, MA, USA) to determine crematocrit[223]. The remaining sample was analyzed for protein concentration using Bio-Rad DC Protein Assay (Bio-Rad Laboratories Hercules, CA, USA).

4.2.6 Oligosaccharide Identification

An additional tube of milk from each dam was collected in which potential differences in oligosaccharide (OS) composition and abundance was determined using mass spectrometry (MS) and gas chromatography (GC). Prior to MS and GC analysis, OS were isolated from the milk and purified according to a previously described method[224] and dried in a speed vacuum centrifuge to a stable powder. To quantify the oligosaccharides' building blocks it was necessary to cleave the oligosaccharides into the monosaccharide components before analysis and also to derivatize the released monosaccharides into volatile molecules. Therefore, methanolysis in conjunction with derivatization by trimethylsilylation was performed following a procedure based on the previously described method[225].

4.2.7 HPLC-Chip/Q-TOF MS

Prior to MS analysis, the dried oligosaccharide samples were reconstituted in 100 µL of nanopure water. MS was performed with an Agilent 6520 accurate-mass Quadrupole-Time-of-Flight (Q-TOF) LC/MS with a microfluidic chip (Agilent Technologies, Santa Clara, CA, USA) as previously described[226]. The microfluidic chip consists of an enrichment column and an analytical column, both packed with porous graphitized carbon, and a nano-electrospray tip. Separation was performed by a

binary gradient of solvent A (3% ACN, 0.1 % formic acid (FA) in water) and solvent B (90% ACN, 0.1% FA in water). The column was initially equilibrated and eluted with a flow rate of 0.3 mL/min for the nanopump and 4 mL/min for the capillary pump. The 65 min gradient was programmed as follows: 2.5-20 min, 0-16% B; 20-30 min, 16-44% B; 30-35 min, 44-100% B; 35-45 min, 100% B; 45-65 min, 0% B (to equilibrate the chip column before the next sample injection). Data were acquired in the positive ionization mode with a mass range of m/z 450 to m/z 2500.

4.2.8 Oligosaccharide Identification

The Molecular Feature Extractor function of Mass Hunter Qualitative Analysis Version B.04.00 (Agilent Technologies) was used to generate a list of deconvoluted masses. All masses were then filtered to be in the range of m/z 450-1500 with a minimum height count of >1000 and typical isotopic distribution of small biological molecules. Only charge states of +1 and +2 were allowed. OS compositions were then determined from the deconvoluted mass list with an in-house program, Glycan Finder, with a mass error tolerance of ≤5 ppm. Distinct OS structures were identified based on accurate mass and retention times compared to previously identified structures. Monosaccharide compositions were further confirmed by MS/MS analysis.

4.2.9 Statistics

All data are presented as mean ± SEM. One-way ANOVA was used to compare the effect of diet on maternal body weight. Two-way ANOVA was used to compare the main effects of diet and sex, and their interaction, on offspring weight. When a main effect of diet or sex was found without an interaction effect, data were pooled by diet or sex and Tukey's post-hoc analysis performed (for diet which has 3 groups). Where a significant interaction effect was identified, all 6 diet groups were compared using Tukey's post hoc analysis. Comparisons between time points for dams were done using repeated measures ANOVA with diet as a between-subjects factor. Comparisons between time points (day 35 and sacrifice) and the gastrointestinal site (cecal vs. fecal) for the

offspring were compared using repeated measures ANOVA with diet and sex as between-subjects factors. When a significant interaction effect was found, estimated marginal means were compared with Bonferroni adjustment. The reference group was not included in the statistical analysis. $P \leq 0.05$ was considered significant. Statistical analysis was performed using IBM SPSS statistics v 19.0 software (Chicago, IL).

Oligosaccharide statistical analyses were performed on the deconvoluted masses corresponding to known OS compositions using the software Agilent Mass Profiler Professional (MPP) version 2.2. In MPP all the compounds were matched and aligned for each retention time (RT). The maximum shift allowed for RT correction was according to the formula: (0.5% of the RT + 0.5 min) with a mass window of 200 ppm. The retention time window allowed for compound-matching was ±0.25 minutes with the addition of ±0.25% of the RT at each time point. Potential contaminants were removed using a filter that retained only compounds present in at least two of the samples and present in at least 25% of the samples within a diet group. The data pre-filtering led to a matrix composed of 295 compounds. Compound intensities were normalized on the median intensity of each compound. This normalization intended to provide equal weight to both low and high abundance peaks for the Principal Component Analysis (PCA). An asymptotic ANOVA (paired conditions) test with the Benjamin Hochberg FDR correction was performed to highlight the differences between the groups. The final results are presented in a three axes PCA plot. PCA was performed using intensity and volume of compounds (compounds were defined as a mass and an RT) across the three different diet groups.

4.3 Results

4.3.1 Body Weight

After one week on the experimental diets, HF dams had significantly lower body weight than HP dams ($P=0.026$) (Table 4.4). Before parturition, HF dams maintained a lower body weight than HP dams ($P=0.008$), and there was a trend for HF dams to have lower body weight than C dams ($P=0.078$). At weaning (three weeks post-partum), HF dams weighed less than HP dams ($P=0.025$).

At weaning there were no sex differences for offspring body weight but HP offspring were heavier than HF offspring (P=0.012). At 5 weeks of age, a sex difference was evident with males weighing more than females although there was no longer a diet effect. Similarly at 14 weeks, just prior to starting the HFS diet, offspring body weight was significantly affected by sex (P=0.001), with males weighing more than females, but not diet (Table 4.5).

4.3.2 Milk

Protein content of the maternal milk samples did not differ between groups (C: 19.4±0.9; HP: 21.0±1.0; HF: 19.8±1.2 mg/µl; P=0.524). Similarly, no differences were seen in crematocrit (C: 16.9±2.0; HP: 15.7±2.1; HF: 20.5±2.5%; P=0.329).

4.3.3 Gas chromatography analysis

A representative GC chromatogram for a control rat milk sample is presented in Figure 4.2. The average amount of the monosaccharides constituting oligosaccharides in the milk samples did not differ between groups (C: 3.4±0.6; HP: 3.2±1.4; HF: 4.0±0.7g/L). The relative abundance of all released monosaccharides in each group is presented in Figure 4.3. Overall, the more abundant monosaccharides composing the free oligosaccharides found in the 3 groups were: sialic acid (NeuAc), followed by D-glucose, D-galactose and *N*-acetylglucosamine. *N*-acetylgalactcosamine and fucose were present but only at the trace level. One representative annotated chromatogram of each of the C, HP and HF milk samples displaying all the peaks corresponding to oligosaccharides is presented in Figure 4.4. The intact oligosaccharides were identified by tandem mass spectrometry.

The PCA plot of the asymptotic ANOVA paired conditions test with the Benjamin Hochberg FDR correction is displayed in Figure 4.5. The first 3 principal components explained nearly 60% of the total variance in the data (PC1: 30.04; PC2:14.56; PC3:5.55). Among the oligosaccharides influencing the clustering, the one with the most significant fold-change among groups is the compound with m/z 546.2029

(P<0.001). This oligosaccharide has a composition of 2 Hex + 1 GlcNAc. The Log Fold change of the HP versus C was 14.5. The Log Fold change of HP versus HF was -6.145. Figure 4.6a shows the Extracted Ion Chromatogram of oligosaccharide with mass 546.2029. The arrow indicates the low abundant isomer at RT of 20.40 min that is only present in the fiber group. The second significant fold-change among oligosaccharides was the compound with mass 634.2189 (P<0.001). Its composition is 2 Hex + Sialic acid (NeuAc). The Log Fold change of HP versus C was 19.5. The Log Fold change of HP versus HF was -0.37. Figure 4.6b shows the Extracted Ion Chromatogram of oligosaccharide with mass 634.2189. The arrow indicates the isomer at RT 13.03 min that is more abundant in the HF group. There were 11 oligosaccharides that were present in all samples and confirmed by mass spectrometry (2hex 1fuc, 3hex, 2hex 1HexNAc, 2hex 1NeuAc, 2hex 1NeuGc, 1hex 1hexNAc 1NeuAc, 3hex 1hexNAc, 2hex 1hexNAc 1 NeuAc, 2hex 2NeuAc, 3hex 1hexNAc 1NeuAC, 4hex 2NeuAc).

Table 4.4. Body weight of dams consuming a C, HP or HF diet during pregnancy and lactation

	Control	High Protein	High Fiber
Pre-conception weight (g)	296.3±6.0[ab]	314.7±6.2[a]	292.1±5.8[b]
Week 3 weight (g)	446.0±12.4[ab]	458.5±12.1[a]	405.9±9.6[b]
Weaning weight (g)	345.3±5.0[ab]	362.1±6.9[a]	335.6±7.2[b]

Values are mean ± SEM with n=8-10 per group. Treatments with different letters are significantly different between diets (p<0.05).

Table 4.5. Body weight of offspring derived from dams consuming a C, HP or HF diet

	Sex	Maternal Diet				2-way ANOVA P-values		
		C	HP	HF	Reference	Diet	Sex	D × S
3 weeks	M	48.2±0.7	47.3±1.0	46.7±1.0	59.5±0.9	0.012	0.230	0.744
	F	46.8±0.7	47.1±0.7	44.6±1.2	59.2±0.6			
5 weeks	M	148.3±3.5	145.7±2.8	148.8±3.8	104.6±3.1	0.668	0.001	0.825
	F	131.1±3.9	128.7±3.5	128.4±4.5	100.1±1.3			
14 weeks	M	549.5±11.1	545.0±7.8	541.1±9.8	553.1±12.5	0.477	0.001	0.537
	F	297.5±6.0	316.1±13.0	292.4±7.0	321.2±9.2			
22 weeks	M	709.2±30.1	674.9±22.3	684.8±19.6	623.9±21.9	0.305	0.001	0.092
	F	384.1±14.2	429.8±22.5	354.7±9.9	360.7±10.2			

Values are mean ± SEM with n=8-10 per group.

Figure 4.2. Gas chromatography profile of trimethylsilyl methyl glycoside derivatives generated after methanolic HCl treatment of a mixture of purified milk oligosaccharides

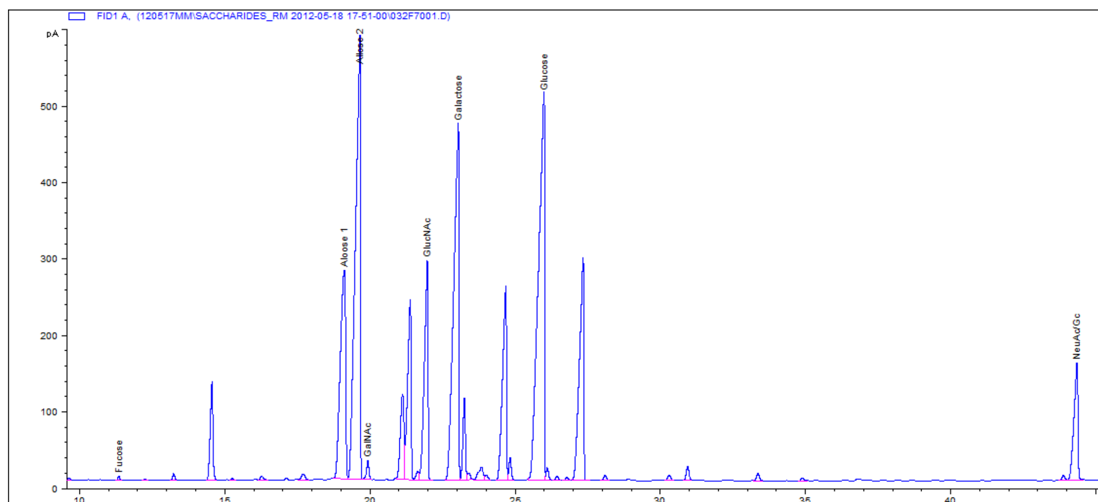

Figure 4.2: Typical gas chromatography profile of the trimethylsilyl methyl glycoside derivatives generated after methanolic HCl treatment of a mixture of purified milk oligosaccharides (sample shown from control dam). The monosaccharides analyzed were fucose, *N*-acetylgalactosamine (GalNAc), *N*-acetylglucosamine (GlucNAc), galactose, glucose, *N*-acetylneuraminic acid (NeuAc), and *N*-glycolylneuraminic acid (NeuGc). Response factors for each monosaccharide were calculated based on D-allose, the internal standard.

Figure 4.3. Relative monosaccharide abundance

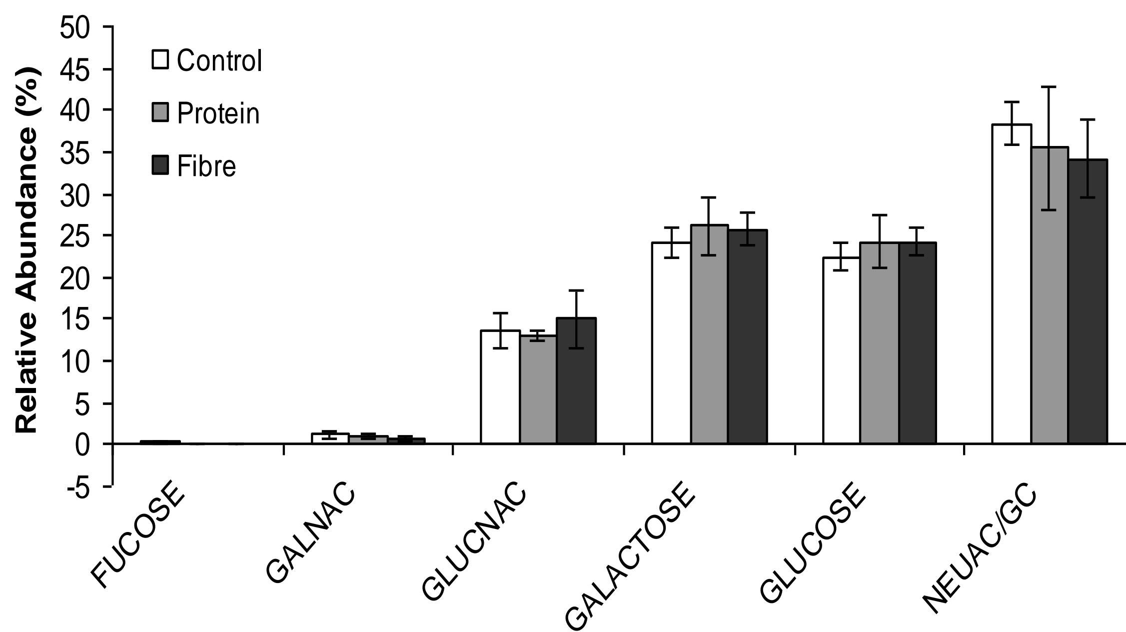

Figure 4.3: Relative abundance of the monosaccharides composing milk oligosaccharides as analyzed by gas chromatography. Values are means± SEM, *n* = 10.

Figure 4.4. Annotated chromatogram generated by tandem mass spectrometry

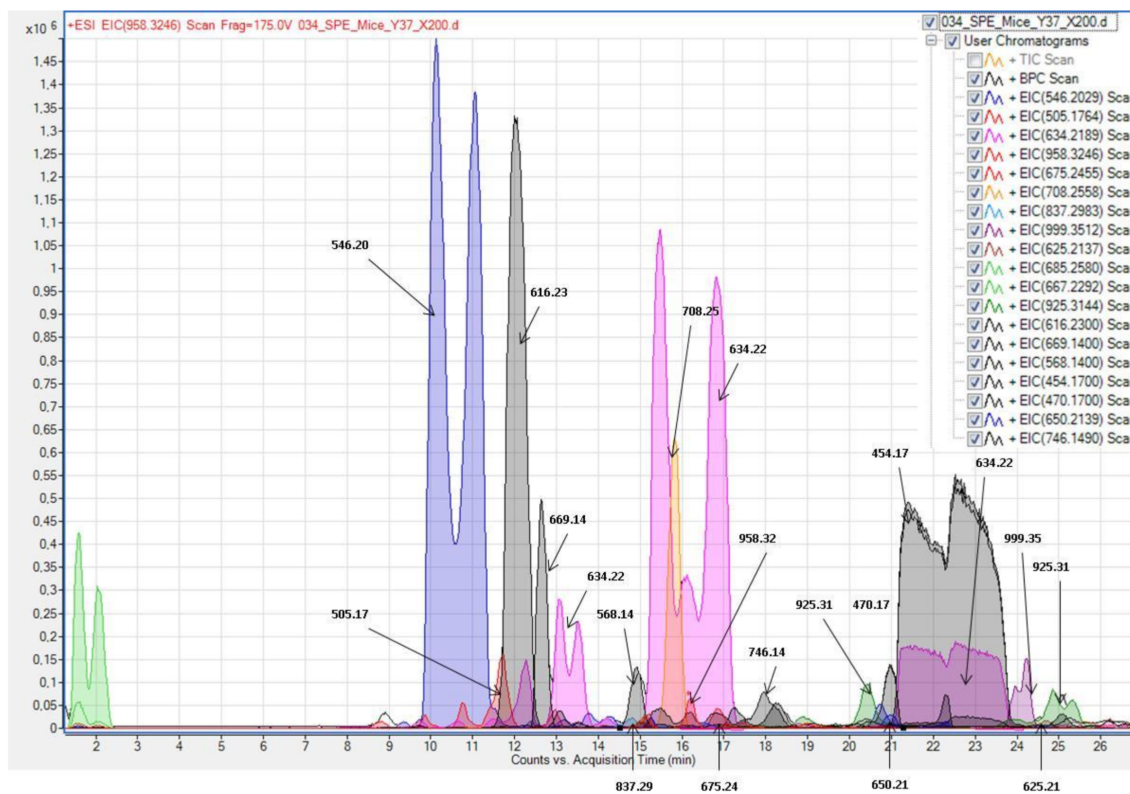

Figure 4.4: Annotated chromatogram of an HF sample displaying all the peaks corresponding to oligosaccharides identified by tandem mass spectrometry.

Figure 4.5. PCA plot grouping of milk from rat dams

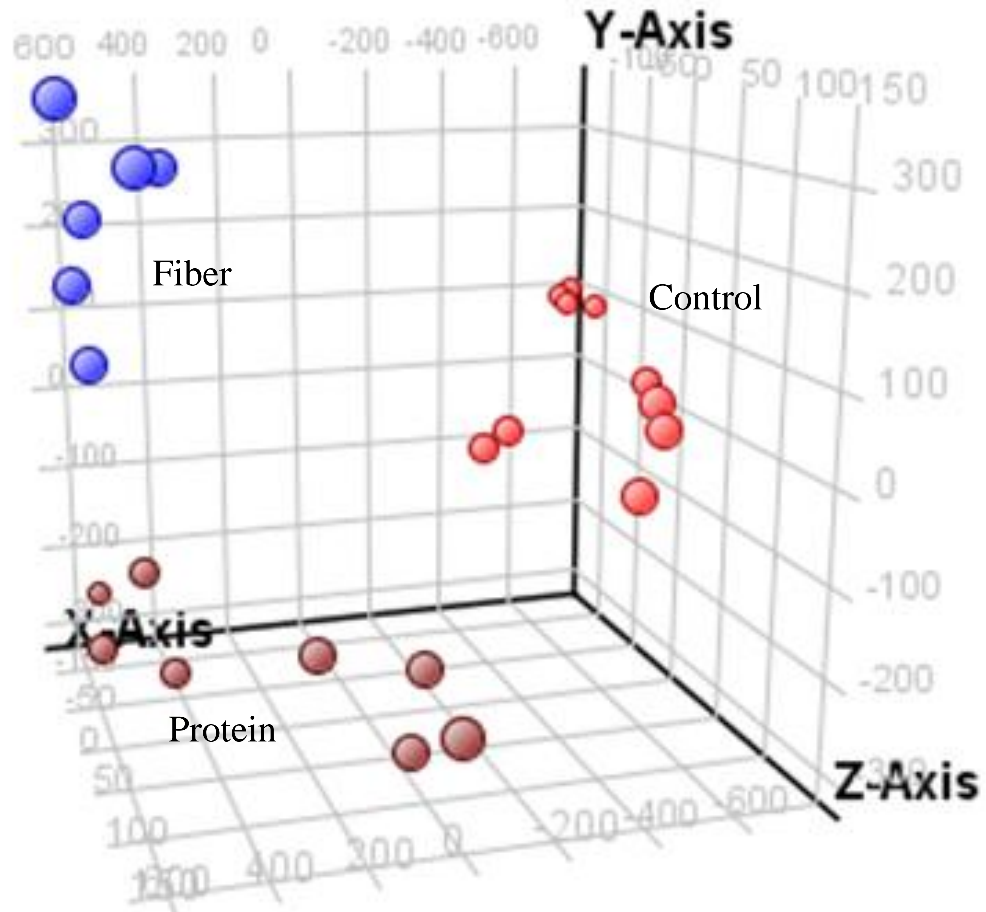

Figure 4.5: Tri-dimensional scatter plot showing grouping of principle component analysis. Scores for the dams milk oligosaccharides from three groups given with one of three nutritionally complete experimental diets: high prebiotic fiber (HF), high protein (HP), or control (C).

Figure 4.6. Extracted ion chromatogram of oligosaccharides

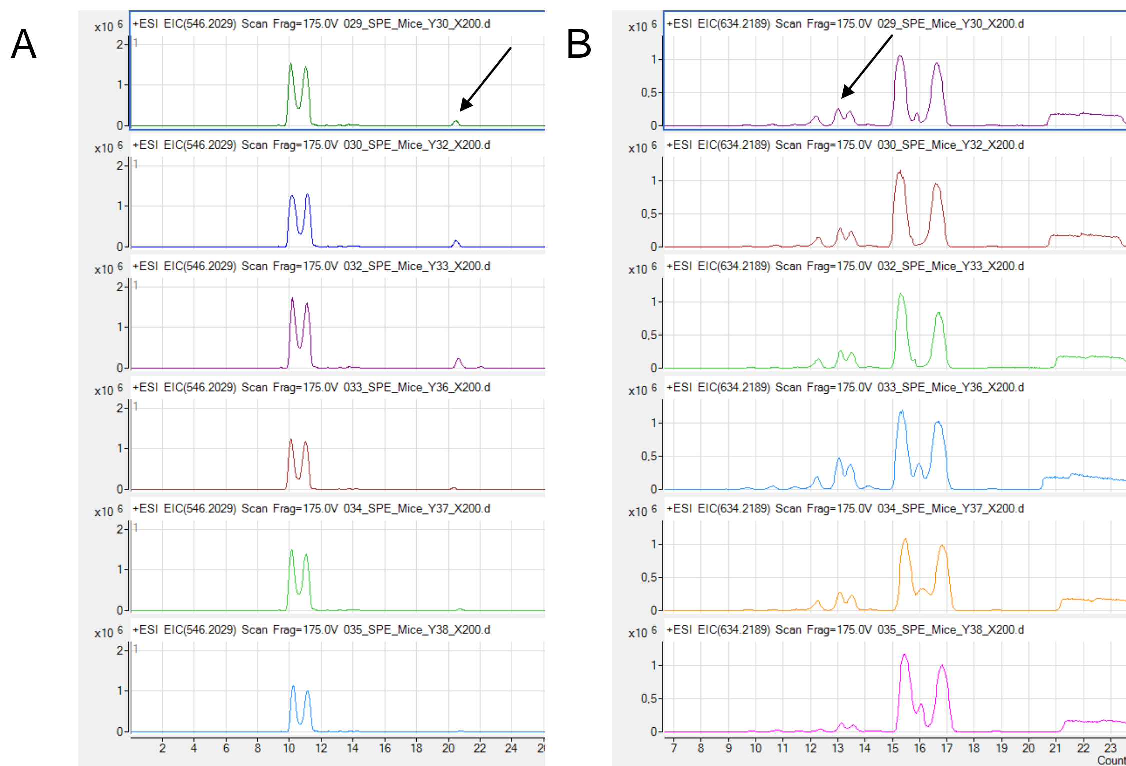

Figure 4.6: (A) Extracted Ion Chromatogram of oligosaccharide with protonated mass 546.2029. Arrows indicate the low abundant isomer at RT of 20.40 min that is only present in the fibre group. The second significant fold-change among oligosaccharides was the compound with protonated mass 634.2189 ($P<0.001$). Its composition is 2 Hex + Sialic acid (NeuAc). The Log Fold change of HP versus C was 19.5. The Log Fold change of HP versus HF was -0.37. (B) Extracted Ion Chromatogram of oligosaccharide with mass 634.2189. Arrows indicate the isomer at RT 13.03 min that is more abundant in the HF group.

4.3.4 Maternal Microbiota

There was a significant effect of time for maternal *Bacteroides* spp.*, C. coccoides, C. leptum, Lactobacillus* spp.*, Enterobacteriaceae*, and *Clostridium* Cluster I, with greater abundance two weeks post-partum than at parturition for all except *Enterobacteriaceae*, which was greater at delivery (P<0.04) (Figure 4.7). A significant effect of diet was seen for *C. coccoides* with HF having greater abundance than HP and C (P<0.01), with the same pattern seen for *Bifidobacterium* spp. (P=0.05). Diet significantly affected *Clostridium* Cluster XI with HP being greater than HF (P<0.01). Both time and diet affected *Methanobrevibacter* (P<0.01) with HF being greater than C and HP at parturition, and parturition being greater than two weeks post-partum.

4.3.5 Offspring Microbiota Change From 5 Weeks to 22 Weeks

Gut microbiota in offspring measured for each diet, sex and time point are shown in Figures 4.8 and 4.9. There were significant interactions between time, sex, and diet for *C. coccoides* and *Bacteroides* spp. with the abundance of *Bacteroides* spp. being greater in female HF1 than female HP1 (P<0.01).

Both time and diet influenced *Lactobacillus* spp., *Bifidobacterium* spp., *C. leptum*, *Clostridium* Cluster I (P=0.015), *Roseburia* spp. and *Methanobrevibacter* (P=0.027). The abundance of *C. leptum* was greater in all diet groups at the younger age of 5 weeks compared to 22 (P=0.01). *Lactobacillus* spp. was greater in C1 and HP1 at 5 weeks compared to 22 (P=0.046). *Bifidobacterium* spp. was greater at 5 than 22 weeks in C1 offspring (P=0.028). *Roseburia* spp. was greater in C1 than HP1 and HF1 at 5 weeks (P<0.02). At 22 weeks *Roseburia* spp. was greater in HP1 than C1 and HF1 (P<0.02). Time and sex significantly affected *Enterobacteriaceae* (P=0.011) and *Methanobrevibacter* (P=0.002).

4.3.6 Offspring Microbiota Change Between Cecal and Fecal

Roseburia spp. abundance was greater in the cecum than distal colon of offspring. Intestinal site, sex, and diet all influenced *Bacteroides* spp. (P=0.042) and *Bifidobacterium* spp. (P=0.017) wherein *Bacteroides* spp. was higher in the cecum than distal colon for all diet groups and in the distal colon was greater in female HP1 than male HP1 (P<0.05). *Bifidobacterium* spp. was greater in the distal colon of HF1 females compared to HP1 females, and was greater in the cecum of HF1 males than HF1 females, HP1 males, and C1 males. *Bifidobacterium* spp. was also significantly greater in the cecum than distal colon for HF1 males (P<0.02).

Both the intestinal site and diet influenced *C. coccoides* and *Lactobacillus* spp. abundance (P=0.046). *C. coccoides* was higher in the cecum of HF1 compared to HP1 and C1; and for all diets was greater in the cecum than distal colon (P<0.01). *Lactobacillus* spp. was greater in HP1 than C1 in the distal colon; and greater in the cecum of HF1 than the distal colon (P<0.05). Both intestinal site and sex influenced *C. coccoides* (P=0.034), *Enterobacteriaceae* (P<0.001), *C. leptum* (P=0.008), *Clostridium* Clusters I (P<0.001) and XI (P=0.003) and *Methanobrevibacter* (P<0.001). *C. coccoides* was greater in the distal colon of males than females, and greater in the cecum than distal colon for both sexes (P<0.04). *Enterobacteriaceae* was greater at both sites in males than females, and was greater in the cecum than distal colon for females (P<0.001).

4.3.7 Endotoxemia

Both diet and sex influenced plasma LPS concentrations (P=0.001). For both HP1 and HF1, plasma LPS was higher in males than females (P<0.001). Female C1 had greater levels than female HP1 and HF1 (Male C: 1047.0±132.4, P: 1176.5±126.8, F: 1092.3±74.8; Female C: 1111.2±70.8, P: 592.7±146.5, F: 383.1±45.0; P<0.01).

Figure 4.7. Dam microbiota at parturition and 14 days post-partum

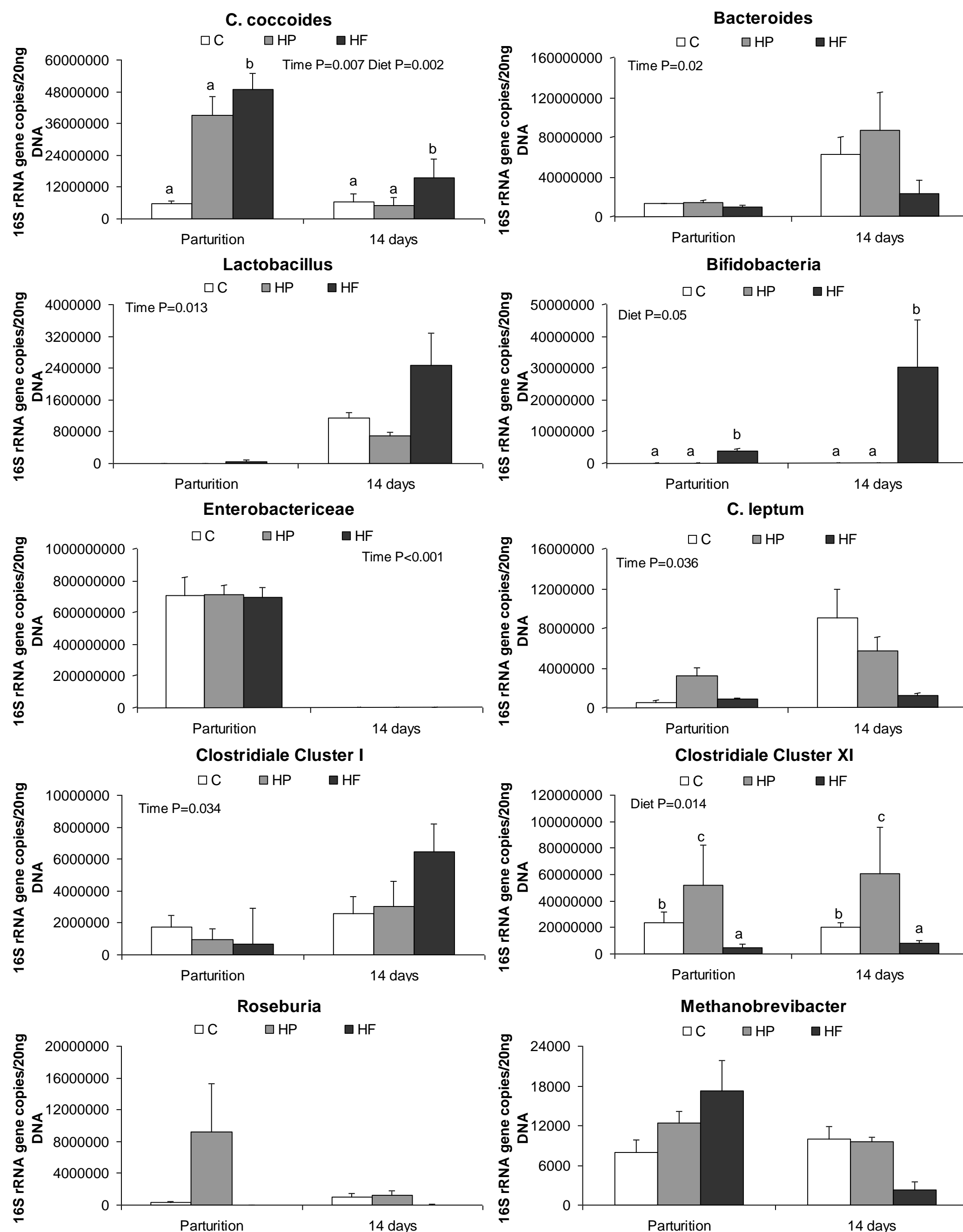

Figure 4.7: Fecal microbiota of dams at parturition and 2 weeks post-partum. Values are means ± SEM, n = 9-10. Data expressed as 16S rRNA gene copies/20ng total genomic DNA.

Figure 4.8. Offspring fecal and cecal microbiota members of the Bacteroidetes, Actinobacteria, Archea and Proteobacteria phyla

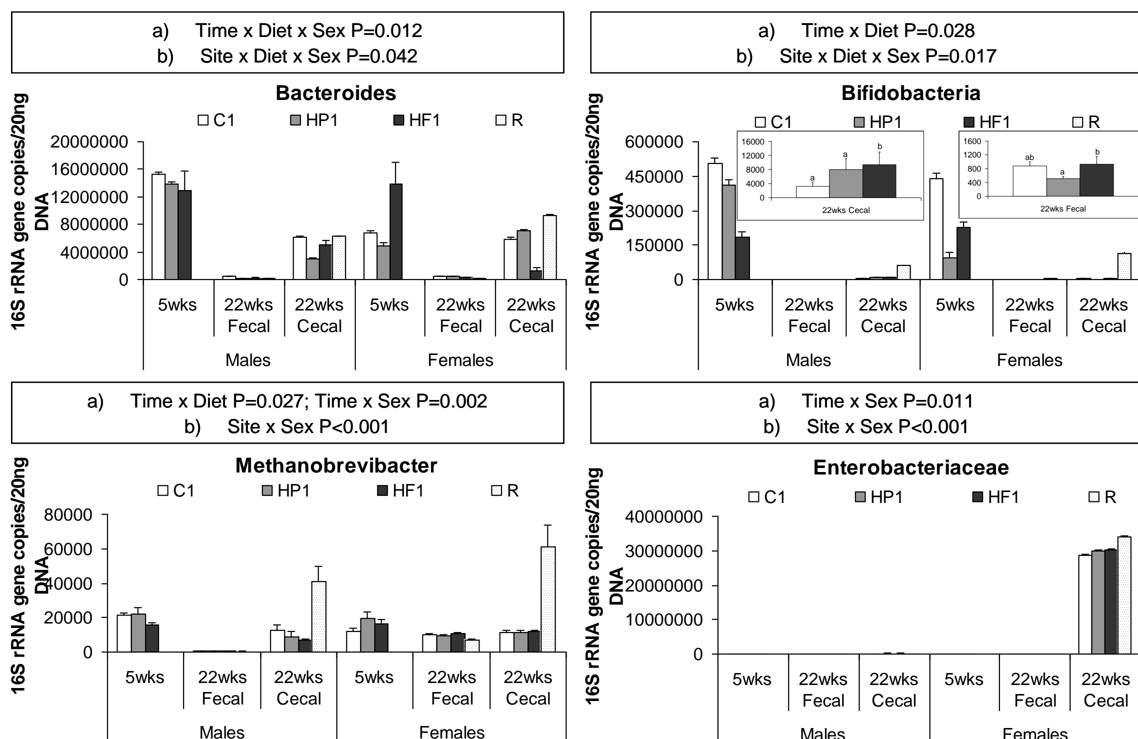

Figure 4.8: Fecal and cecal microbiota members of the Bacteroidetes, Actinobacteria, Archea and Proteobacteria phyla of offspring derived from dams consuming C, HP or HF. Values are means ± SEM, n = 9-10. Data expressed as 16S rRNA gene copies/20ng total genomic DNA. Fecal contents were analyzed at 5 and 22 weeks of age, cecal contents were analyzed at 22 weeks of age. a) indicates significant differences in fecal contents between 5 and 22 weeks; b) indicates significant differences between fecal and cecal contents at 22 weeks.

Figure 4.9. Offspring fecal and cecal microbiota members of the Firmicutes phylum

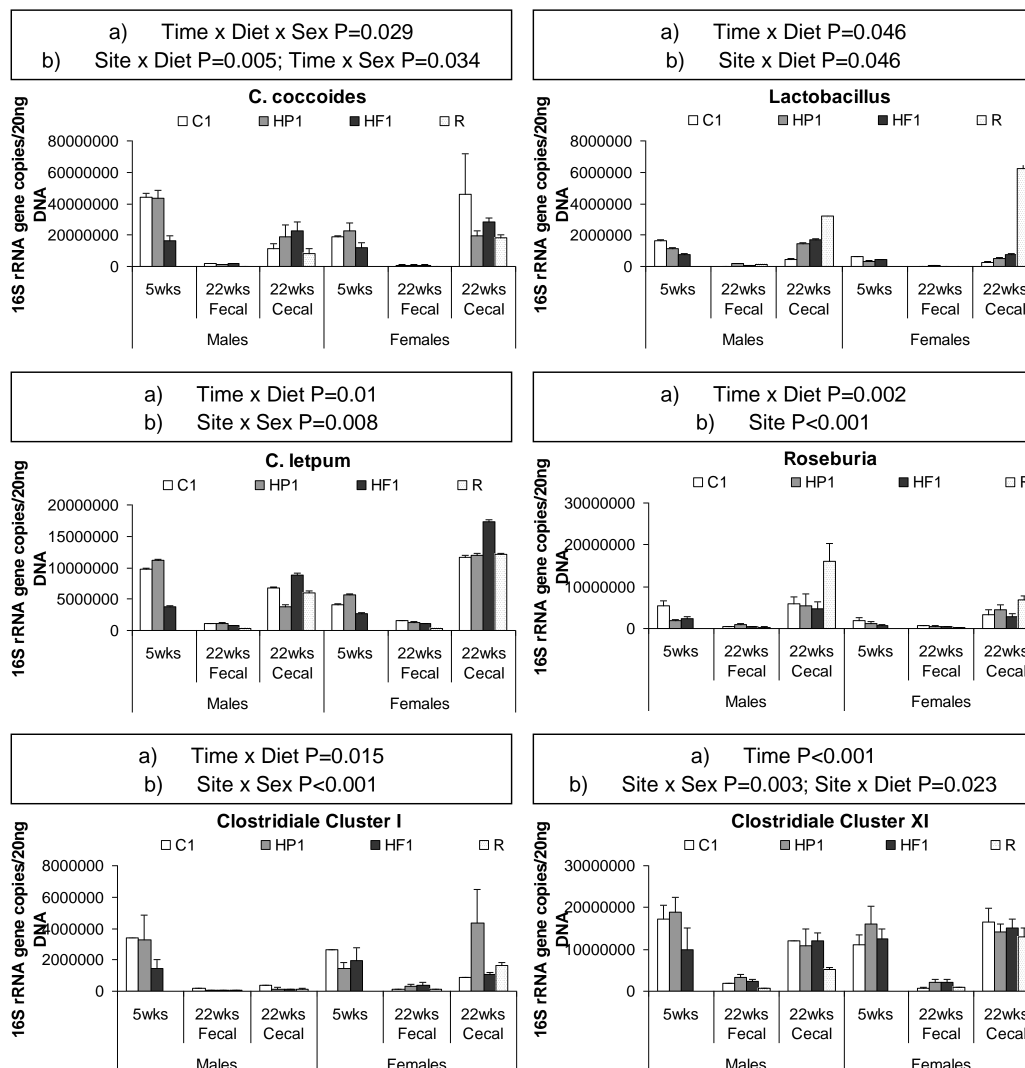

Figure 4.9: Fecal and cecal microbiota members of the Firmicutes phylum of offspring derived from dams consuming C, HP or HF. Values are means ± SEM, n = 9-10. Data expressed as 16S rRNA gene copies/20ng total genomic DNA. Fecal contents were analyzed at 5 and 22 weeks of age, cecal contents were analyzed at 22 weeks of age. a) indicates significant differences in fecal contents between 5 and 22 weeks; b) indicates significant differences between fecal and cecal contents at 22 weeks.

4.4 Discussion

The infant gut microbiota plays an important role in early health, and when not interrupted due to illness or substantial prolonged dietary changes, will remain quite stable in adulthood based on the microbiota established in early life (approximately 3 years of age in humans[227]). The gut microbiota acquired during infancy is important in training the infant's immune system[228] and helps protect against pathogens[229]. In this study all pups were dam-reared and we sought to identify differences in dam and pup microbiota that could be related to maternal diet composition. Given that the gut microbiota changes drastically when the infant gut is no longer exposed to maternal milk[216], it is possible that the differential microbiota acquired by the pups in our study due to maternal diet ultimately affected the persistence of such beneficial species such as bifidobacteria into adulthood.

Maternal gut microbiota is an important factor in the establishment of offspring microbiota[149]. From birth to 2 weeks post-partum, all bacterial groups examined in the dams increased in abundance or did not change, except *Enterobacteriaceae* which decreased. *Enterobacteriaceae* has been shown to increase in response to inflammation and throughout pregnancy[26, 169], which is in itself a state of insulin resistance and associated with inflammation[230]. As expected based on previous studies, our mixture of inulin and oligofructose increased bifidobacteria in the dams consuming the HF diet, a change which is frequently reported to have beneficial effects on metabolic health[25, 231]. An increase in the butyrate producing *C. coccoides* was also seen, which has been observed in mice fed oligofructose and is associated with insulinaemia[163, 219, 232]. Abundance of the *Clostridium* Cluster XI, from which opportunistic pathogens can emerge, was also decreased similar to that observed with probiotic feeding[233].

To gain a better understanding of the changes in offspring microbiota in response to maternal diet and a postnatal high fat diet challenge we examined fecal microbiota in offspring at 5 and 22 weeks and cecal microbiota at 22 weeks. The response to the diet challenge in adulthood is important given evidence that consuming a high fat diet

decreases total bacterial numbers, the *E. rectale-C. coccoides* group and *Bifidobacterium* spp., while increasing proportions of Bacteroidales and Clostridiales[167, 169]. In our study, fecal *Bifidobacterium* spp. abundance was found to be greater in HF1 females than HP1 females at 22 weeks, and male cecal *Bifidobacterium* spp. was greater in HF1 than C1 and HP1 animals. *Bifidobacterium* spp. are the predominant species in the gut during suckling but have been shown to momentarily disappear at weaning and not recover to the same degree[216]. Recently it has been shown in humans that offspring microbiota are more similar to that of the mother during her first trimester than the third, this being true for the offspring at 4 years of age more so than at 1 or 6 months of age[26]. As we did not have fecal samples from the first week of pregnancy in our dams it is difficult to know the full extent of the influence of maternal microbial profiles early in pregnancy on the offspring microbiota.

Passage through the gastrointestinal tract, reflected in our comparison of cecal contents to expelled fecal matter showed that the relative abundance of most bacteria decreased the more distal the site. This site difference may be indicative of an increased diversity of bacteria in the cecum versus the distal colon, or could reflect other differences between the sites such as pH or SCFA concentrations. For example, differences in microbial profiles and diversity between stool and mucosa and at various anatomical sites in the gastrointestinal tract have been previously reported in humans[234]. Such differences could at least partially be related to our differences in cecal and fecal matter as fecal pellets are made up of the nonadherent populations as well as shed mucosal bacteria[26, 235]. Given the importance of the establishment of the indigenous or autochthanous bacteria in the naïve gut[236], further work examining the influence of maternal diet on these communities in offspring is warranted.

Offspring sex had a fairly profound influence on the proportions of microbiota in the offspring, with some also being dependent on maternal diet exposure. Clear differences have been shown in the serum metabolite profiles of male and female mice raised in a conventional setting[235]. This is in contrast to germ free mice where no sex differences were found leading to the suggestion that the gut microbiota may have a sex-specific role to play in metabolism that could be linked to testosterone[235]. This may

help explain some of the anthropometric and satiety hormone differences between male and female animals we have previously reported with the same high protein and fiber maternal diets[221]. Specifically, body weight and fat mass were higher in the female offspring of the high protein versus high fiber dams whereas no differences in body weight or fat mass were seen in the male offspring[221]. There was also a significant sex effect for fasting and total area under the curve for ghrelin and fasting GIP, with females having higher levels than males[221]. Ober *et al*., [237] have proposed that sex-specific genetic architecture influences phenotypes and that interactions between genotype and sex contribute to the sexual dimorphism seen in many common diseases.

Understanding that early diet exposure has an important impact on gut microbiota, we examined the composition of maternal milk to determine if any compositional changes could explain differences in offspring microbiota. Milk samples were distinguishable between the different diet groups, with notable increases in two oligosaccharides, Hex + 1 GlcNAc and 2 Hex + Sialic acid (NeuAc), in milk from HP and HF dams. Other unidentified glyco-conjugates are likely the other distinguishing factors among the three diet groups. Oligosaccharides in human milk (HMO) have been shown to increase beneficial bacteria in the gut, such as *Bifidobacterium* spp., *Bacteroides* spp., and Eubacteria/clostridia[238, 239]. The neutral oligosaccharides, such as Hex + 1 GlcNAc have been found to be important for the development of the intestinal microbiota and are strongly related to the microbial profiles found in breast-fed infants[240]. Acidic oligosaccharides, which include 2 Hex + Sialic acid, have been found to be utilized by *B. infantis* and may help prevent adherence of pathogens to the epithelial surface[241]. These acidic structures also prevent pathogenic bacteria from adhering to the epithelial surface due to structural similarities[242]. The persistence of *Bifidobacterium* spp. in HF1 offspring and *Lactobacillus* spp. in HF1 and HP1 offspring shows greater stability in these species into adulthood, even after consuming a high fat diet. The resilience in these species could relate to the increased presence of bifidobacteria in HF dams, conferred to offspring from birth, through milk consumption and the shared environment of the cage, and/or may relate to the oligosaccharide exposure as this was similar in HP and HF dams.

As demonstrated by Cani et al., a HFS diet is expected to increase intestinal permeability and plasma LPS and thereby trigger inflammation or metabolic endotoxemia[243]. Both HP1 and HF1 females had lower levels of plasma LPS than C1 females at study termination. The higher levels measured in C1 females could be related to the differences seen in the oligosaccharides of the maternal milk, as it has been previously observed that HMO can increase binding of bifidobacteria to intestinal cells which regulates tight junction proteins, increasing intestinal barrier function[231]. Endotoxemia is negatively correlated with *Bifidobacterium* spp. [167]. HF1 females had greater numbers of *Bifidobacterium* spp. than HP1 females. It has previously been reported that the microbial community of the offspring is very similar to that of the mother since many bacterial species are conferred on the offspring at birth[217]. Bacterial transfer may also occur *in utero*[244] and through maternal milk[214]. While HP dams had increases in the same oligosaccharides as HF dams, the HP1 offspring did not show the same increases in *Bifidobacterium* spp. This could relate to differences in autochthonous versus allochthonous bacterial communities and how they regulate the intestinal barrier as previously the mucosal microbiota have specifically been found to play a role in nutrient exchange and innate immunity[245].

These results also highlight the importance of bacterial transfer from dam to offspring during pregnancy, potentially from umbilical cord blood and amniotic fluid, and during lactation due to bacterial transfer from the gut to mammary gland[153]. Different strains of bifidobacteria also have different capabilities for using oligosaccharides as growth substrates[246]. Recent work shows that individual human milk oligosaccharides (HMO) are fermented to a greater or lesser extent by microbiota and that fecal inocula from formula fed infants fermented numerous substrates more rapidly than breast fed infant inocula[246]. Given the distinct microbiota profiles found in our HF and HP dams, it is possible that the offspring's utilization of the two oligosaccharides found to be increased in this study with the HF and HP diets could have differed. The oligosaccharides may have had a greater impact on early gut development and gut closure, and prevented a "leaky gut" even with exposure to the HFS diet, decreasing the plasma levels of LPS in HF1 and HP1. It would be interesting to have

measures of gut development factors such as GLP-2 at an earlier age to coincide with microbiota at 5 weeks to determine if there were differences in this trophic factor. LPS has also been correlated with poor glucose handling, inflammation, weight and fat gain[167]. As previously reported, our HP1 animals had improved glucose handling as measured by HOMA-IR score[221]. HF1 females had decreased body weight and percent body fat at study termination[221], which is associated with increased *Bifidobacterium* spp. and decreased plasma LPS.

In conclusion, altering maternal diet composition, while not changing protein or fat content, did alter the predominance of certain oligosaccharides in maternal milk. These differences were associated with increases in *Bifidobacterium* spp. in HF1 offspring which persisted to 22 weeks, and stability of *Lactobacillus* spp. in HP1 and HF1 offspring even after high fat feeding, indicating a certain degree of protection may have been conferred to the offspring in regards to their metabolic health.

Chapter Five: Dietary mis-match and re-match: Impact of diet composition in adult rats is dependent on maternal diet[5]

5.1 Introduction

While *in utero* the fetus is subjected to numerous cues about the environment it will encounter in postnatal life. The fetus is completely dependent on maternal nutrient supply for normal growth and development and therefore many of the environmental cues it encounters are linked to maternal diet. When a mismatch occurs between the nutritional environment predicted by the fetus and the actual environment encountered postnatally, the risk of developing chronic diseases, such as metabolic syndrome or type 2 diabetes, hypertension or cardiovascular disease, is increased[2]. More recent evidence also shows detrimental effects on appetite regulation and glucose and lipid metabolism in response to nutritional mismatch[247]. This programming effect has often been examined in terms of an adverse maternal environment, such as energy or protein restriction, drug treatment, or a maternal high fat diet which is then mismatched in offspring with provision of a normal, control diet or a high fat diet, either at weaning or in adulthood. Even small degrees of mismatch can have negative consequences on offspring, although in general the greater the mismatch the greater the consequences[38]. While it is typical for a weaning diet high in fat to have negative effects on offspring health regardless of maternal diet, there are some metabolic markers that have been shown to improve with consistent dietary exposure from the pre- to post-natal period. Offspring of high fat-diet fed dams who were also fed high fat diet at weaning had decreased plasma triglycerides and improved endothelial function compared to their littermates given a control diet[38]. This finding highlights the importance of the predictive adaptive response and provides

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[5] Portions of this work have been presented in part at: Canadian Student's Health Research Forum, June 2011. *Re-matching adult offspring to maternal diet high in prebiotic fiber impacts gut hormone response.*
Obesity, October 2011. *Re-matching offspring to maternal high protein or prebiotic fiber improves insulin sensitivity*. Obesity, *19*(S1): S89.
Canadian Nutrition Society, May 2012. *Pre-natal prebiotic fiber exposure and responsiveness to the same diet after a high fat high sucrose dietary challenge.*

justification for examining whether a healthy maternal diet, matched in the offspring could in fact result in the lowest disease risk.

A diet high in prebiotic fiber has been shown to have beneficial effects on satiety, food intake, triglyceride accumulation and glycemic control[117, 118, 200, 248]. We have previously shown that prenatal exposure via a maternal diet high in prebiotic fiber has beneficial effects on offspring when challenged with a high fat high sucrose diet (HFS) in adulthood, resulting in decreased weight gain and adiposity[221]. With postnatal exposure, wherein animals were weaned onto a HF diet, challenged in adulthood with the HFS diet and then re-matched to the HF diet, reduced adiposity was observed compared to HP animals[117]. Conversely, when the maternal diet was high in protein, offspring were predisposed to excessive weight gain and adiposity when challenged with HFS in adulthood[221]. Similar detrimental effects on body weight also occurred with exposure to a high protein (HP) diet at weaning and throughout growth followed by a HFS diet in adulthood[113]. However, when pups consumed an HP diet after HFS exposure, glycemic response, as well as percent body fat was normalized, showing potential value in re-matching to an expected nutritional environment[117]. It is possible that re-matching to a maternal HP diet after a dietary HFS challenge in adulthood could also have beneficial effects on the offspring.

The purpose of this study was to examine the impact of dietary patterns represented by mismatching and re-matching between prenatal and postnatal diets in rats. Our first aim was to examine how consumption of a HFS diet in adulthood affected control rats and whether the impact of a transient HFS diet could be mitigated by returning to a control diet following HFS diet. The second aim was to evaluate the impact of re-matching offspring to their maternal diets after HFS exposure in adulthood. Thirdly, we compared the impact of the experimental diets on offspring when they were re-matched to their maternal diet, versus being exposed to the diet for the first time after HFS diet exposure in adulthood.

5.2 Methods

5.2.1 Ethical approval

The University of Calgary Animal Care Committee approved the experimental protocol which was conducted in accordance with the Guide for the Care and Use of Laboratory Animals.

5.2.2 Animals and Diets

Thirty-seven virgin Wistar dams were obtained from Charles River (Montreal, QC, Canada) and housed in a temperature and humidity controlled facility with a 12-h light/dark cycle. After 1 week of acclimatization, animals were given one of three nutritionally complete experimental diets: high prebiotic fiber (HF) (21.6% wt/wt, 1:1 ratio of oligofructose and inulin; 13.73 kJ/g), high protein (HP) (40% wt/wt; 15.74 kJ/g), or control (C) (based on AIN-93G; 15.74 kJ/g). All maternal diets were mixed in house using ingredients purchased from Dyets, Inc. (Bethlehem, PA, USA); the detailed composition can be found in Tables 5.1 through 5.4[112]. An additional 15 dams consumed AIN-93G throughout the study to form a reference group. Dams consumed the diets for one week prior to being bred with male Wistar rats in wire-bottomed cages. Following the identification of a copulation plug, dams were housed individually and continued to consume their assigned experimental diet (C, HF, or HP) until the pups were weaned. Dams were weighed weekly, and food intake was measured throughout week 2 of pregnancy.

Pups were weighed on the day after birth, and litters then culled to 10 pups with equal numbers of males and females where possible. Offspring were weighed weekly for the remainder of the study. Food intake was also measured for 5 consecutive days out of every 20 days by subtracting the weight of the cup and diet from the previous days' weight. At weaning (3 weeks), 1 male and 1 female pup were randomly selected from each litter to continue in the study until 28 weeks of age. By selecting one male and one female from each litter we examined n=10 individual rats per sex that were not all from

one litter, minimizing the effect of any single dam. Pups were weaned onto AIN-93G control diet[187]. Offspring were then switched to AIN-93M (15.07 kJ/g) for maintenance at 10 weeks of age. At 14.5 weeks of age, offspring were fed a high fat, high sucrose (HFS) diet (19.26 kJ/g) for 8 weeks. The HFS diet composition can be found in Table 5.1. Thirteen males and 13 females were kept on AIN-93M as a reference group (R). This reference group, matched for age and sex to the intervention groups, provides a standard of normal growth in these rats. After 8 weeks on HFS, rats were re-matched to the diet of their respective dams for 6 weeks and identified as C1, HP1 or HF1. Reference animals were randomized to the high fiber (RF) or high protein (RP) diet. Three males and four females continued to consume the HFS diet for the final 6 weeks (H). A summary of the experimental design and diet succession can be found in Figures 5.1 and 5.2.

5.2.3 Oral glucose tolerance test and tissue sampling

Four days before the end of the study, rats were fasted overnight and an oral glucose tolerance test performed (OGTT). Blood was sampled from the tip of the tail in conscious rats followed by an oral glucose gavage (2 g/kg). At 15, 30, 60 and 90 min post-glucose gavage, additional blood was sampled from the tail and immediately analyzed using a blood glucose meter (Accu-Chek Blood Glucose Meter, Laval, QC). One day prior to study termination rats underwent a DXA scan (Hologic ODR 4500; Hologic Inc.) while lightly anaesthetised using isoflurane. Hologic QDR software for small animals was used to determine lean and fat mass. A second OGTT for satiety hormone analysis was performed at the time of terminal tissue collection. After an overnight fast, rats were anaesthetized with isoflurane and a fasting cardiac blood sample was taken. Rats were then given 50% dextrose (wt/vol) by oral gavage at a dose of 2 g/kg. At 15, 30, 60 and 90 min post-gavage, another cardiac blood sample was taken, according to our previous work, while rats were anaesthetized[188]. Blood was collected in tubes containing diprotinin-A (0.034 mg/ml blood; MP Biomedicals, Irvine, CA); Sigma protease inhibitor (1 mg/ml blood; Sigma Aldrich, Oakville, ON, Canada) and Roche Pefabloc (1mg/ml of blood; Roche, Mississauga, ON, Canada) and then

centrifuged at 1600×g for 12 min at 4ºC. Plasma was stored at -80ºC until analysis. The OGTT was a terminal procedure and after the 90 min blood collection rats were killed via over-anaesthetisation and aortic cut. The liver, stomach, small intestine, cecum and colon were weighed, a sample snap frozen in liquid nitrogen and stored at -80ºC.

5.2.4 Plasma Analysis

A Milliplex Rat Gut Hormone kit (Millipore, St. Charles, MO) and Luminex instrument were used to measure ghrelin (active), insulin, amylin (active), leptin, glucose-dependent insulinotropic polypeptide (GIP) (total) and peptide tyrosine tyrosine (PYY) (total). An ELISA was used to measure active GLP-1 (Millipore, St. Charles, MO). Fasting concentrations of non-esterified fatty acids (NEFA) were measured using an enzymatic colorimetric assay according to manufacturer instructions (Wako Diagnostics, Richmond, VA). Homeostasis model assessment of insulin resistance (HOMA-IR) was calculated from fasting insulin and fasting glucose.

5.2.5 Hepatic Triglyceride Analysis

Triglyceride content of the liver was quantified using 25mg of tissue according to the manufacturer guidelines of the GPO reagent set (Pointe Scientific Inc., Lincoln Park, MI).

5.2.6 RNA Extraction and Real-Time PCR

Total RNA was extracted from the stomach, small intestine, colon and liver using TRIzol reagent (Invitrogen, Carlsbad, USA). Reverse transcription was performed with an input of 1 μg of total RNA using the 1st strand cDNA synthesis kit for RT-PCR (Invitrogen, Carlsbad, CA USA) with oligo d(T)15 as a primer. The cDNA was amplified using primers synthesized by the University of Calgary Core DNA Services (Calgary, AB, Canada) and analyzed by real time PCR. Primer sequences for Acetyl Co-A Carboxylase (ACC), Fatty Acid Synthase (FAS) and sterol regulatory element binding

protein-1c (SREBP1c), glucose-6-phosphatase and AMP-activated protein kinase alpha-1 (AMPKα1) were according to our previous work[112]. A melt curve showed the melting point of the PCR product of interest. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was verified as a suitable housekeeping gene for the tissues of interest and GAPDH primers included as an internal control in the reactions. The $2^{-\Delta CT}$ method [ΔCT = CT (gene of interest) – CT (reference gene)] was utilized for the data analysis where threshold cycle (CT) indicates the fractional cycle number at which the amount of amplified target reaches a fixed threshold[189]. The ΔCT is the difference in threshold cycles for the gene of interest and GAPDH.

5.2.7 Statistical Analysis

All data are presented as mean ± SEM. Data collected from the dams was analyzed with one-way ANOVA with Tukey's post hoc analysis. In offspring, a two-way ANOVA was used to compare the main effects of diet and sex and their interaction. Only when a significant interaction effect was identified were all 6 or 4 groups, as applicable, compared using a one-way ANOVA with Tukey's post hoc analysis. A Bonferroni correction was applied and $P \leq 0.05$ was considered significant. Statistical analysis was performed using SPSS v 19.0 software (Chicago, IL).

Table 5.1. Growth and HFS diet compositions

Ingredient	Control	High Protein	High Fiber	High Fat/Sucrose
Cornstarch (g/kg)	397.5	197.5	253.3	47.5
Casein (g/kg)	200.0	400.0	174.5	140.0
Dyetrose (g/kg)	132.0	132.0	115.8	-
Sucrose (g/kg)	100.0	100.0	87.7	512.5
Soybean Oil (g/kg)	70.0	70.0	61.4	100.0
Lard (g/kg)	-	-	-	100.0
Alphacel (g/kg)	50.0	50.0	43.9	50.0
AIN-93M Mineral Mix (g/kg)	35.0	35.0	30.7	35.0
AIN-93-VX Vitamin Mix (g/kg)	10.0	10.0	8.8	10.0
L-Cystine (g/kg)	3.0	3.0	2.6	2.0
Choline bitartrate (g/kg)	2.5	2.5	2.2	3.0
Inulin (g/kg)	-	-	109.6	-
Oligofructose (g/kg)	-	-	109.6	-

Table 5.2. Growth and HFS macronutrient compositions

% of Total Calories	Control	High Protein	High Fiber	High Fat/Sucrose
Carbohydrate	63.80	44.67	64.16	49.75
Protein	19.45	38.59	19.26	11.13
Fat	16.74	16.74	16.58	39.12

Table 5.3. Maintenance diet compositions

Ingredient	Control	High Protein	High Fiber
Cornstarch (g/kg)	465.7	205.7	378.7
Casein (g/kg)	140.0	400.0	140.0
Dyetrose (g/kg)	155.0	155.0	155.0
Sucrose (g/kg)	100.0	100.0	100.0
Soybean Oil (g/kg)	40.0	40.0	40.0
Alphacel (g/kg)	50.0	50.0	50.0
AIN-93M Mineral Mix (g/kg)	35.0	35.0	35.0
AIN-93-VX Vitamin Mix (g/kg)	10.0	10.0	10.0
L-Cystine (g/kg)	1.8	1.8	1.8
Choline bitartrate (g/kg)	2.5	2.5	2.5
Inulin (g/kg)	-	-	107.5
Oligofructose (g/kg)	-	-	107.5

Table 5.4. Maintenance macronutrient composition

% of Total Calories	Control	High Protein	High Fiber
Carbohydrate	73.98	48.07	74.41
Protein	14.11	40.00	13.87
Fat	9.99	10.00	9.82

Figure 5.1. Re-matching experimental design

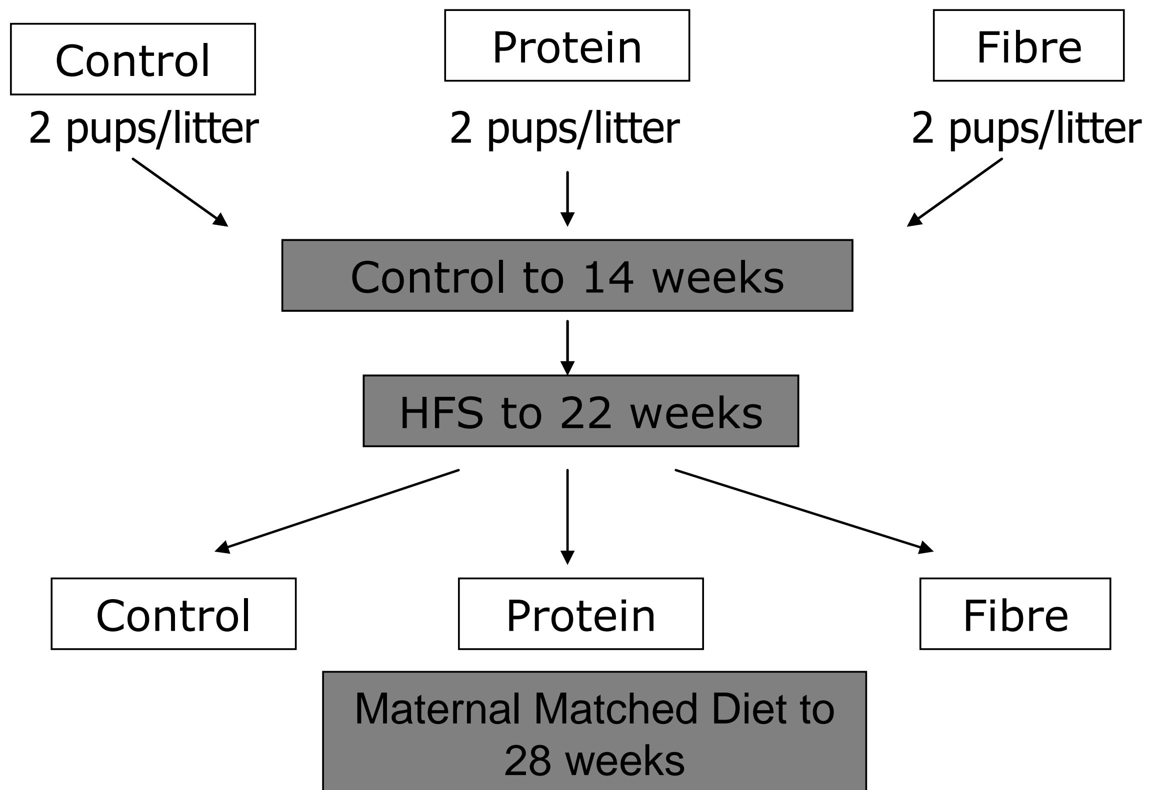
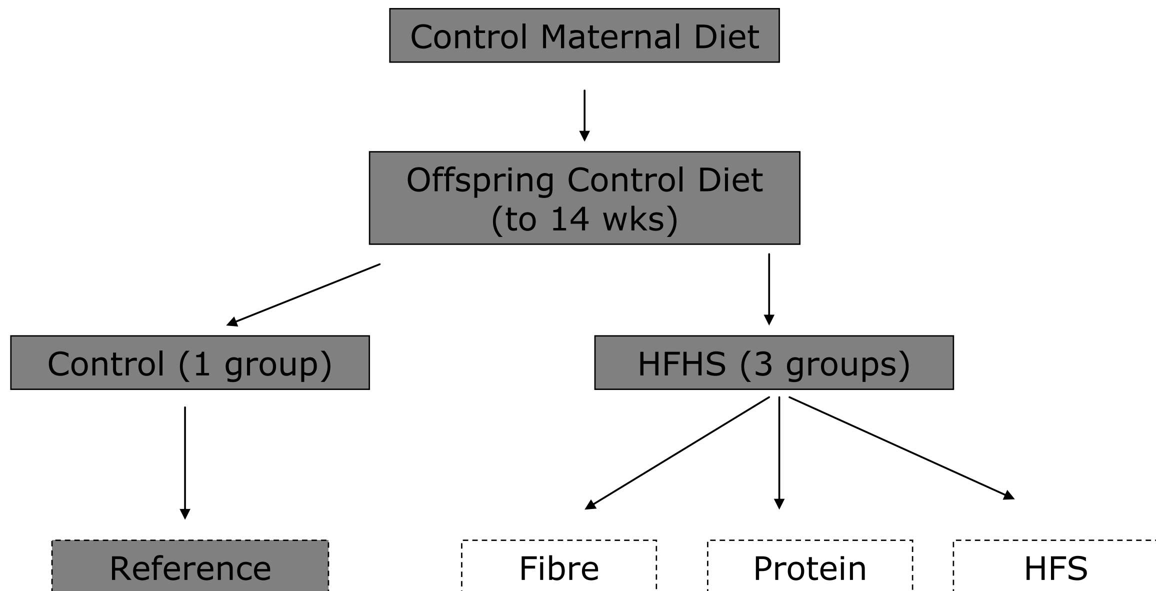


Figure 5.2. Re-matching Reference Groups



5.3 Results

5.3.1 Effect of HFS Exposure and Reversibility

The following results describe the outcomes in offspring that were never exposed to the HFS diet (i.e. AIN-93 throughout) (R), re-matched to the AIN-93M for 6 weeks following the HFS challenge (C1), or continued on HFS for an additional 6 weeks post-HFS challenge (H).

5.3.1.1 Growth and Energy Intake

The growth trajectory of the pups to the end of the HFS period (22 weeks) has been previously reported[221]. For the control rats that either continued on AIN-93M (R), switched back to AIN-93M following HFS (C1) or continued on HFS (H), there was a significant effect of time but not diet on body weight ($P<0.001$) (Figure 5.3). Energy intake at 24 weeks was greater in C1 than H ($P=0.025$). At 27 weeks, energy intake was greater in R than C1 and was also greater in males than females ($P=0.037$, $P<0.001$) (Figure 5.3).

5.3.1.2 Body Composition

There were no statistical differences in body weight or percent body fat between the three groups of offspring from control dams; namely C1, R or H (Figure 5.3). Diet had a significant effect on stomach weight, small intestine length, cecum weight, bone mineral density (BMD) and lean mass measured in offspring at 28 weeks of age (Table 5.5). Stomach weight ($P=0.003$), BMD ($P=0.004$) and lean mass ($P=0.009$) were greater in C1 while small intestine length ($P=0.026$) and cecum weight ($P<0.001$) were greater in R. There was also a significant sex effect on these parameters, with males having higher values than females ($P<0.001$).

5.3.1.3 Plasma Satiety Hormones and Blood Glucose

The interaction between diet and sex affected fasting concentrations and area under the curve (AUC) for GLP-1, as well as fasting amylin and insulin (Table 5.6, Figure 5.4). GLP-1 was higher in R than C1 females (P=0.03), fasting amylin was higher in male H than C1 and R (P<0.001), and fasting insulin was higher in male C1 than R and H (P=0.006) (Table 5.6). Diet had a significant effect on fasting and AUC glucose, for amylin and ghrelin AUC and for fasting GIP. C1 rats had higher fasting glucose than R and H rats (P=0.001) and greater glucose AUC (P<0.001) and amylin AUC (P<0.001) than R rats. Ghrelin AUC (P=0.021) and fasting GIP (P=0.023) were greater in R than C1.

5.3.2 Offspring Re-Matched to Maternal HP, HF or C

The following results describe the outcomes in rats re-matched to the diet they were exposed to *in utero*, either control (AIN-93M) (C1), HF (HF1), or HP (HP1).

5.3.2.1 Growth and Energy Intake

Among animals being re-matched to HF, HP or C diets, body weight was significantly affected by the interaction of time and diet (P<0.001) (Figure 5.5). There was also an independent effect of sex with males weighing more than females (P<0.001). At weeks 26 and 27, C1 weighed more than HF1. When rats were re-matched to their maternal diet following the HFS challenge, C1 rats lost weight over the first 3 weeks but then increased thereafter. HF1 rats lost weight throughout the re-matching period resulting in a final body weight that was significantly lower than that at week 23. Energy intake was affected by the interaction of diet and sex at 24 weeks of age with male HP1 consuming more energy than male HF1 (P=0.034). At 27 weeks there was a significant sex effect with males consuming more energy than females (P<0.001) (Figure 5.5).

5.3.2.2 Body Composition

HF1 animals had lower body weight and percent body fat than C1 and HP1 animals at 28 weeks of age (P=0.009; P<0.001) (Figure 5.5). Small intestine length, colon length and mass, and cecum weight were greater in HF1 than C1 and HP1 animals (P<0.001) (Table 5.5). Colon mass was also greater in HP1 than C1 animals (P<0.001). Kidney weight was greater in HP1 than HF1 and C1 (P=0.037). Fat mass was greater in C1 than HF1 (P=0.012) with a trend for HP1 to have greater fat mass than HF1 (P=0.056). Sex had a significant effect on the above parameters, with males having greater values than females (P<0.001).

5.3.2.3 Plasma Satiety Hormones and Blood Glucose

The interaction of time, diet and sex affected insulin (P=0.036) and PYY levels (P=0.020). Insulin was greater in C1 males than females at 0, 15 and 90 min. In males at 0, 15 and 90 min, C1 had higher insulin than HP1 and HF1, at 60 min. In females at 30, 60 and 90 min, C1 was greater than HF1. PYY was greater in HF1 males than females at all time points, and greater in HF1 than HP1 and C1 at all time points. GLP-1 was affected by diet and sex with male HF1 having higher concentrations than HP1, and female HF1 having higher levels than C1 (P=0.032). The interaction of time and sex influenced ghrelin (P=0.022) with females showing a decrease over the course of the OGTT and males having higher plasma levels until 90 minutes. GIP was affected by the interaction of time with diet (P=0.043) as at 60 minutes HP1 plasma levels were greater than HF1, at 90 minutes C1 plasma levels were greater than HF1 levels. Significant differences over the course of the OGTT were only seen in C1 (0 vs. 15 and 90 min; 60 vs. 90 min) and HP1 offspring (0 vs. 60 minutes). C1 animals had greater leptin levels than HF1 (P<0.001) (Figure 5.6).

Glucose and GIP AUC were greater in C1 than HF1 animals at 28 weeks of age (P=0.026, P=0.001, respectively) (Figures 5.6). C1 also had greater AUC for insulin than HF1 and HP1 (P<0.001). HF1 animals had greater AUC for GLP-1 and PYY than C1 and HP1 (P<0.001) (Figure 5.6). Diet and sex had interaction effects on leptin AUC

(P=0.033) with C1 being higher in males than HP1 and HF1 and greater in C1 and HP1 males than females (Figure 5.6). The interaction of diet and sex also affected HOMA-IR scores, which were higher in male C1 rats than HF1 and HP1 rats (P=0.012) (Table 5.6).

5.3.2.4 Liver Triglyceride

Diet and sex independently and significantly affected liver triglyceride levels (P=0.001). Liver triglycerides were higher in males than females, and lower in HF1 than C1 and HP1 animals (Table 5.6).

5.3.2.5 Hepatic Gene Expression

Diet had a significant effect on expression of hepatic SREBP1c, ACC, FAS, and AMPKα1 (P<0.03) with HP1 animals having greater mRNA levels of SREBP1c than both HF1 and C1, and increased mRNA levels of ACC and AMPKα1 than HF1 and increased FAS compared to C1. Sex had a significant effect on ghrelin, SREBP1c, FAS mRNA levels with males higher than females (P<0.03). The interaction of diet and sex affected PGC1α (P<0.01) and glucose-6-phosphatase mRNA levels (P<0.02) with HP1 females having greater levels than C1 and HF1 (Table 5.7).

5.3.3 Re-matched versus Un-matched Offspring

The following results describe the outcomes in rats that were exposed *in utero* to the HF or HP diet versus those that did not have maternal exposure to the diet (HF1 vs. RF; HP1 vs. RP).

5.3.3.1 Fiber Diet Comparison

Body weight was significantly affected by the interaction of time and maternal diet (P=0.035) with an independent effect of sex (P<0.001). Reference animals (RF) had greater weight than re-matched animals (HF1) at all timepoints. HF1 animals lost weight between 23 and 25 weeks. The RF animals lost weight over the entire 6 week re-

matching period which resulted in significantly lower weight between week 23 and all subsequent weeks; and between week 24 and all subsequent weeks (Figure 5.7). At week 24 there were independent effects of diet and sex on energy intake with HF1 animals consuming more than RF animals ($P=0.045$) and males consuming more than females (Figure 5.7).

Animals exposed *in utero* to the HF diet (HF1) had lower final body weight than the reference animals (RF) whose mothers consumed control diet throughout pregnancy and lactation ($P<0.03$) (Figure 5.7). HF1 also had increased colon length ($P=0.002$) and mass ($P<0.001$), mass also being greater in males than females ($P=0.005$) (Table 5.5), fasting glucose ($P=0.001$) and amylin ($P<0.001$) (Table 5.6), and area under the curve for glucose ($P<0.02$) (Figure 5.8). RF animals had greater liver ($P=0.023$) and cecum weight ($P=0.006$), bone mineral density ($P=0.05$) and lean mass ($P=0.05$) (Table 5.5) compared to HF1 animals. In females, RF animals had greater naso-anal length than HF1 ($P=0.006$) and there was a trend for greater naso-anal length in male HF1 versus RF ($P=0.059$). The interaction of maternal diet and sex affected HOMA-IR scores ($P<0.001$) with male HF1 animals having higher scores than RF, while in HF1 animals males had a higher score than females ($P<0.001$). Fasting leptin was affected by the interaction of maternal diet and sex wherein levels in HF1 males were higher than HF1 females ($P=0.019$) and in males there was a trend for HF1 animals to have higher fasting values than RF ($P=0.058$) (Table 5.6).

5.3.3.2 Protein Diet Comparison

There was no significant difference in body weight or percent body fat between animals re-matched to a high protein diet (HP1) and those exposed to it for the first time at 22 weeks of age (RP) (Figure 5.7). After switching to the HP diet, the interaction between maternal diet and sex affected energy intake at weeks 24 and 27 with male HP1 consuming more than RP but the reverse occurred for females ($P<0.001$) (Figure 5.7). In reference animals there was greater liver ($P=0.033$) and cecum weight ($P<0.001$), small intestine length ($P=0.021$), bone mineral density ($P=0.01$), lean mass ($P=0.033$) (Table 5.5) compared to HP1 animals. HP1 animals had greater colon mass ($P=0.006$), length

(P=0.039) and fasting glucose (P=0.001) than reference animals. Liver triglyceride concentration was affected by the interaction of diet and sex, being greater in RP males than females (P=0.039) (Table 5.6).

Figure 5.3. Physical measures of control animals after HFS-feeding

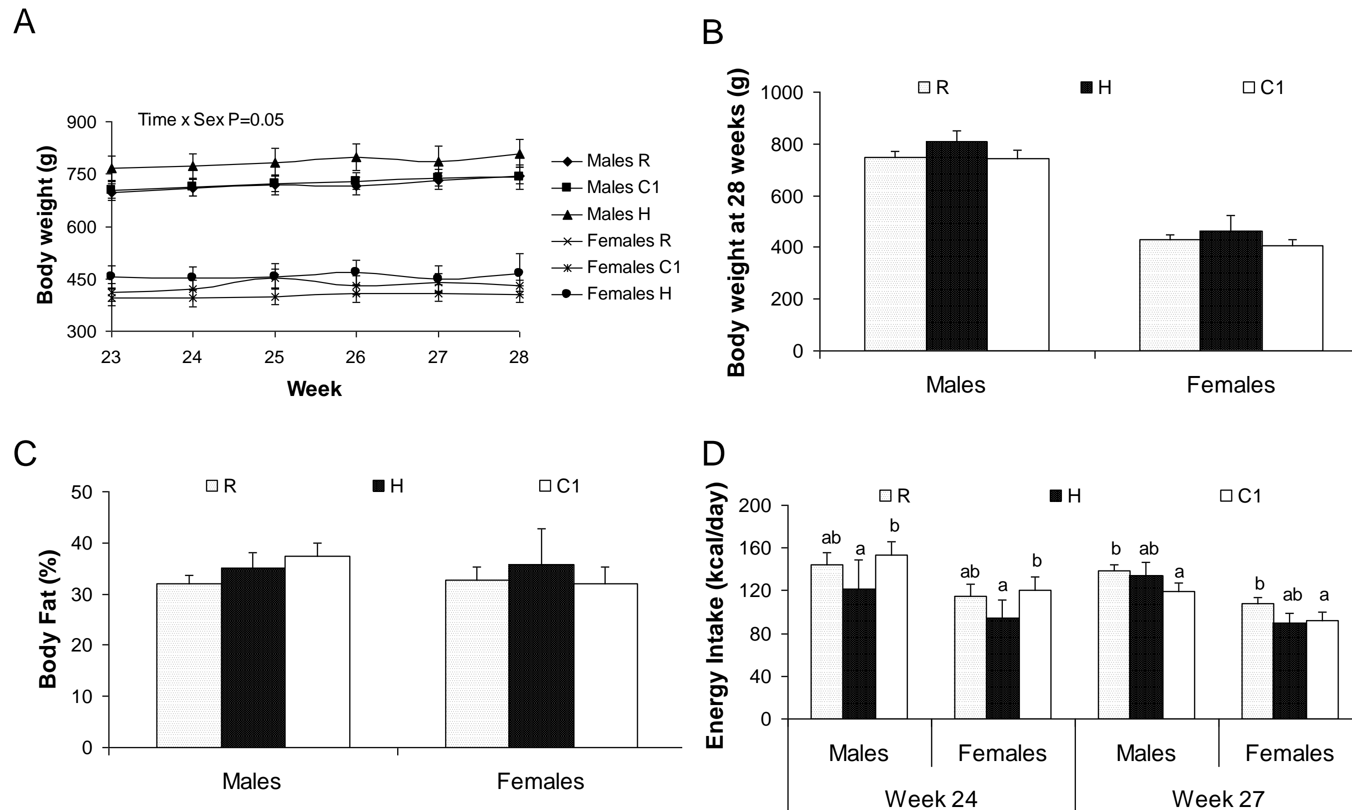


Figure 5.3: A) Post-HFS body weight of males and females. B) Final body weight of males and females. C) Percent body fat of males and females at 28 weeks. D) Energy intake at 24 and 27 weeks. Means with differing letters are significantly different. Values are means ± SEM, n = 4 – 12.

Figure 5.4. Glucose and satiety hormone AUC over a 90 minute OGTT of control animals

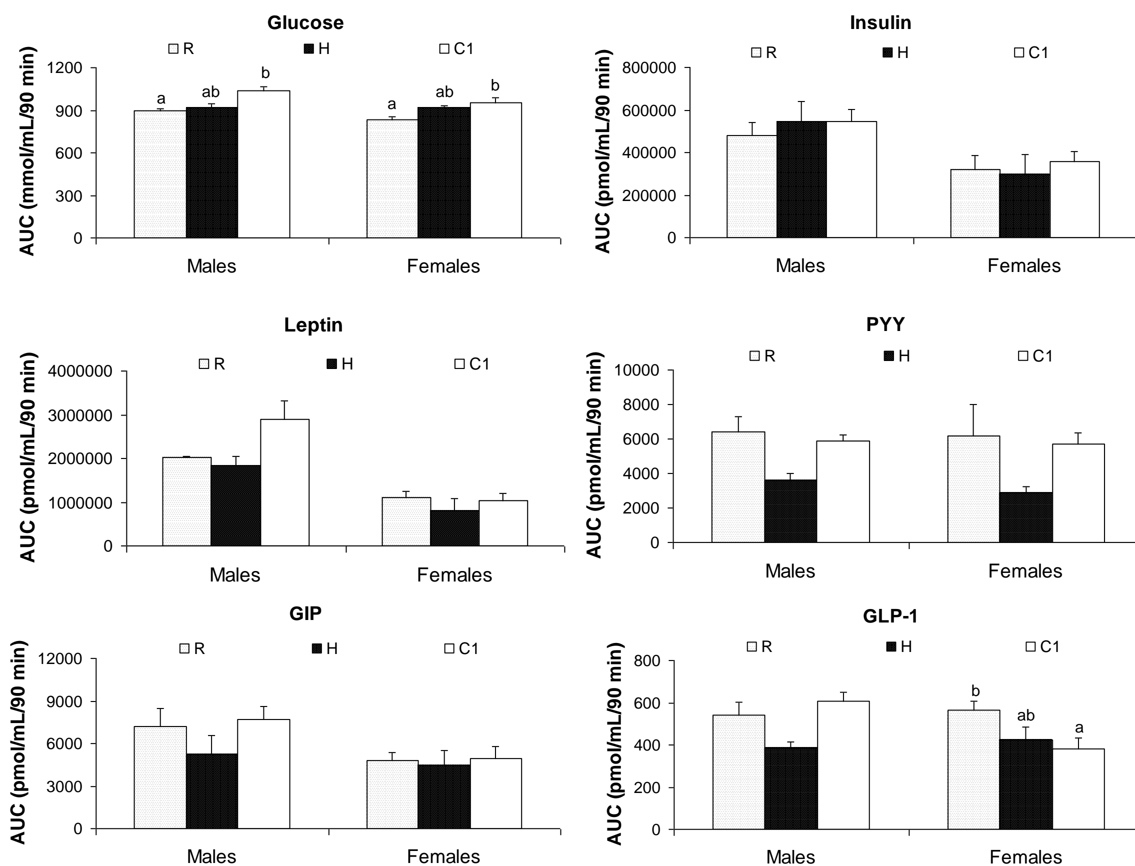


Figure 5.4. Area under the curve for glucose, insulin, leptin, GLP-1, PYY and GIP over a 90 minute OGTT at 28 weeks. Values are means ± SEM, n = 4-12. Treatments with different letters are significantly different between diets.

Figure 5.5. Physical measures of re-matched animals after HFS-feeding

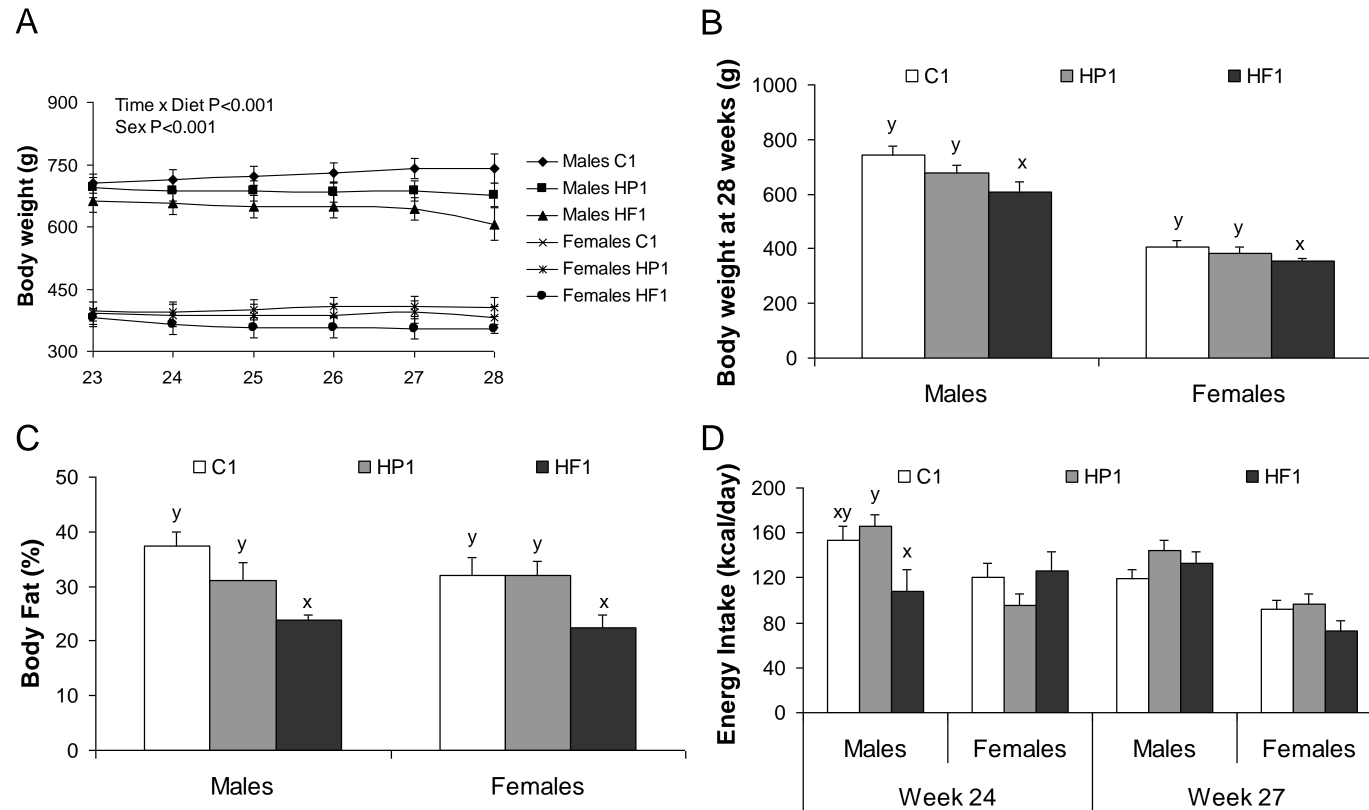


Figure 5.5: A) Post-HFS body weight of males and females. B) Final body weight of males and females. C) Percent body fat of males and females at 28 weeks. D) Energy intake at 24 and 27 weeks. Means with differing letters are significantly different. Values are means ± SEM, n = 8 – 12.

Figure 5.6 Glucose and satiety hormone AUC over a 90 minute OGTT of re-matched animals

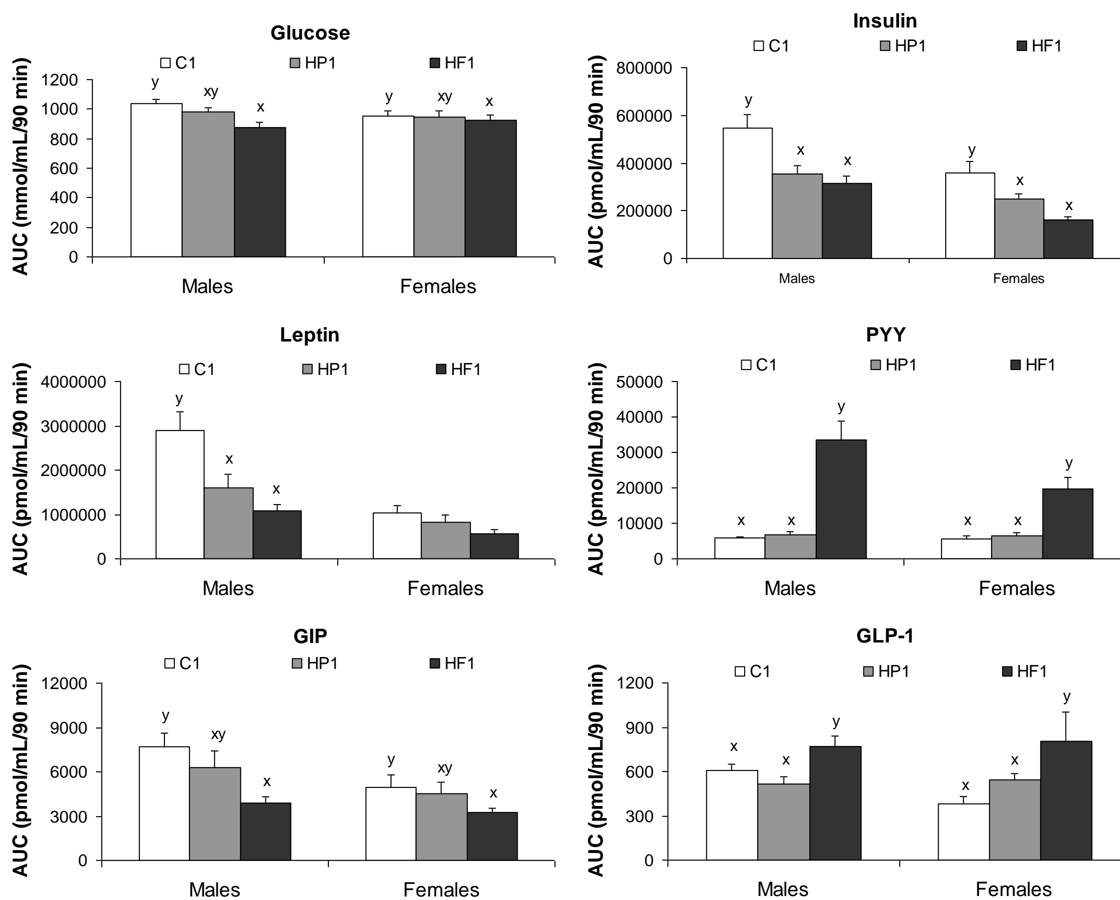


Figure 5.6. Area under the curve for glucose, insulin, leptin, GLP-1, PYY and GIP over a 90 minute OGTT at 28 weeks. Values are means ± SEM, n = 8-12. Treatments with different letters are significantly different between diets.

Figure 5.7. Physical measures of re-matched versus un-matched animals after HFS-feeding

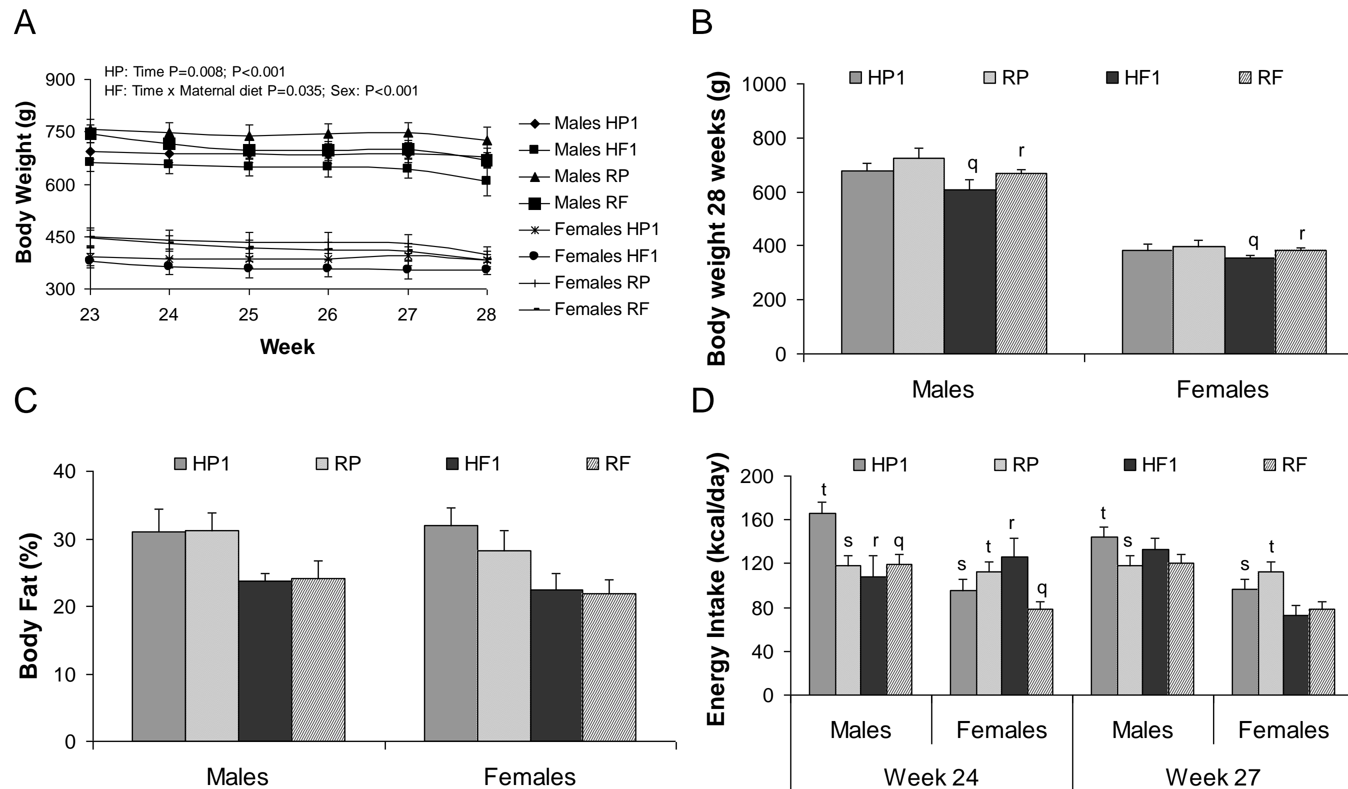


Figure 5.7: A) Post-HFS body weight of males and females. B) Final body weight of males and females. C) Percent body fat of males and females at 28 weeks. D) Energy intake at 24 and 27 weeks. Means with differing letters are significantly different; superscripts [q,r] indicate significant differences between HF1 and RF; superscripts [s,t] indicate significant differences between HP1 and RP. Values are means ± SEM, n = 8 – 12.

Figure 5.8. Glucose and satiety hormone AUC over a 90 minute OGTT of re-matched versus un-matched animals

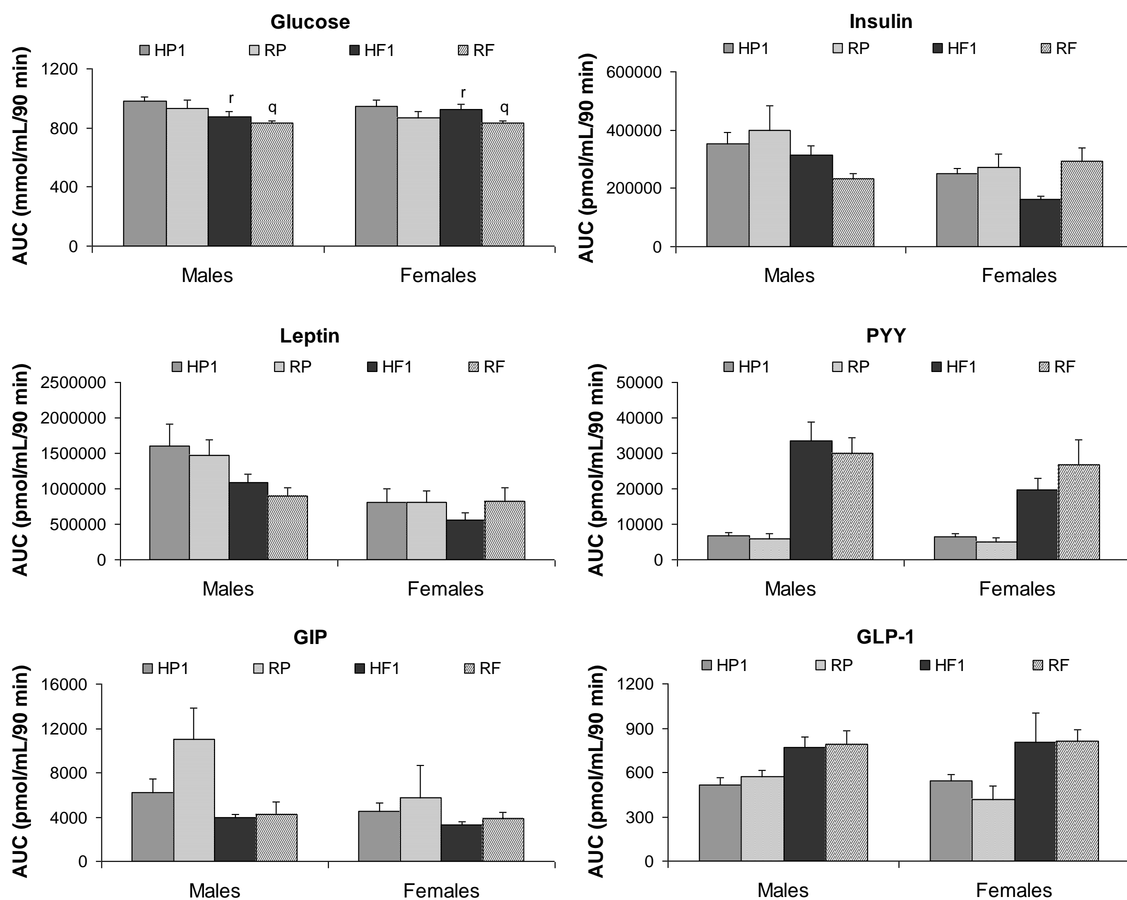


Figure 5.8. Area under the curve for glucose, insulin, leptin, GLP-1, PYY and GIP over a 90 minute OGTT at 28 weeks. Values are means ± SEM, n = 8-12. Treatments with different letters are significantly different between diets.

Table 5.5. Physical characteristics of offspring at 28 weeks

		Diet Group						
		R	H	C1	HP1	HF1	RP	RF
Liver (g)	M	18.8±0.9	19.0±1.0	17.3±0.7	16.1±0.5^{s}	15.5±0.7^{q}	18.1±0.9^{t}	16.7±0.5^{r}
	F	9.0±1.2	10.2±1.1	9.8±0.8	9.5±0.6^{s}	8.9±0.4^{q}	10.5±0.5^{t}	10.6±0.6^{r}
Stomach (g)	M	3.3±0.1^{b}	3.1±0.2^{b}	2.8±0.1^{a}	2.7±0.1	2.6±0.1	3.1±0.2	2.9±0.2
	F	1.9±0.2^{b}	2.4±0.3^{b}	1.9±0.1^{a}	2.0±0.1	2.0±0.1	2.1±0.1	2.1±0.1
Small Intestine (cm)	M	134.8±2.2^{b}	133.8±1.3^{ab}	125.7±2.4^{ax}	130.5±1.5^{sx}	143.8±2.7^{y}	135.0±2.0^{t}	115.3±15.7
	F	104.6±2.2^{b}	122±5.5^{ab}	116.8±3.7^{ax}	115.0±3.4^{sx}	122.8±2.5^{y}	120.5±1.4^{t}	114.0±10.5
Small Intestine (g)	M	8.2±0.24	8.6±0.13	7.9±0.2	8.1±0.4	8.7±0.4	9.0±1.0	6.5±0.9
	F	5.4±0.64	6.3±0.41	6.8±0.3	6.1±0.4	6.1±0.4	6.4±0.29	5.7±0.53
Cecum (g)	M	1.5±0.04^{b}	0.63±0.36^{a}	1.01±0.1^{ax}	1.07±0.1^{sx}	3.2±0.3^{qy}	1.4±0.07^{t}	3.0±0.57^{r}
	F	1.0±0.19^{b}	0.88±0.29^{a}	0.8±0.1^{ax}	0.74±0.06^{sx}	2.3±0.3^{qy}	1.1±0.13^{t}	3.1±0.2^{r}
Colon (cm)	M	21.1±0.43	23.3±0.85	21.6±0.7^{x}	23.5±0.7^{tx}	26.1±0.9^{ry}	21.7±0.65^{s}	19.7±2.7^{q}
	F	16.4±1.9	20.5±1.3	20.0±0.7^{x}	19.6±0.3^{tx}	24.8±0.4^{ry}	20.0±0.47^{s}	22.8±0.6^{q}
Colon (g)	M	1.6±0.05	1.9±0.1	1.8±0.1^{x}	2.1±0.1^{y}	2.4±0.2^{rz}	1.7±0.1	0.96±0.29^{q}
	F	1.1±0.13	1.3±0.06	1.3±0.05^{x}	1.5±0.1^{y}	2.0±0.1^{rz}	1.33±0.09	1.65±0.06^{q}
Kidneys (g)	M	3.5±0.1	3.8±0.2	3.6±0.1^{x}	4.0±0.1^{y}	3.4±0.1^{x}	4.0±0.1	3.5±0.1
	F	1.9±0.2	2.4±0.3	2.3±0.1x	2.4±0.1y	2.1±0.1x	2.4±0.1	2.3±0.8
BMD (g/cm2)	M	0.19±0.002^{b}	0.19±0.006^{b}	0.18±0.003^{a}	0.18±0.002^{s}	0.19±0.003^{q}	0.19±0.002^{t}	0.20±0.004^{r}
	F	0.17±0.002^{b}	0.17±0.004^{b}	0.17±0.002^{a}	0.16±0.002^{s}	0.17±0.001^{q}	0.17±0.002^{t}	0.18±0.001^{r}

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Length	M	28.4±0.16	28.8±0.32	28.5±0.19	28.9±0.16^{t}	28.6±0.34^{rq}	28.3±0.19^{s}	28.0±0.0^{rq}
	F	23.6±0.27	23.8±0.32	24.0±0.17	24.0±0.41^{t}	23.5±0.19^{q}	23.7±0.15^{s}	24.1±0.1^{r}
Lean Mass	M	503.4±10.0^{ab}	526.2±36.7^{b}	457.3±14.2^{a}	459.4±11.8^{s}	491.0±18.8^{q}	491.4±14.6^{t}	503.4±14.9^{r}
	F	285.3±6.6^{ab}	288.7±17.2^{b}	270.7±10.5^{a}	257.8±14.9^{s}	270.7±6.6^{q}	281.2±8.1^{t}	297.5±8.1^{r}

Values are means ± SEMs, *n* = 4 – 12. Superscripts ^{a,b,c} indicate significant differences between R, H, and C1; ^{x,y,z} indicate significant differences between C1, HP1, HF1; ^{q,r} indicate significant differences between HF1 and RF; ^{s,t} indicate significant differences between HP1 and RP. R, reference group never exposed to HFS; H, high fat reference group continued on HFS an additional 6 weeks; C1, animals re-matched to AIN-93M after HFS challenge; HP1, animals re-matched to HP after HFS challenge; HF1, animals re-matched to HF after HFS challenge; RP, reference animals fed HP after HFS challenge; RF, reference animals fed HF after HFS challenge.

Table 5.6. Fasting blood glucose, plasma satiety hormones, HOMA-IR and liver triglycerides in adult offspring re-matched to maternal diet

		Diet Group						
		R	H	C1	HP1	HF1	RP	RF
Glucose (mmol/l)	M	4.98±0.08[a]	4.88±0.45[a]	5.65±0.28[b]	6.38±0.29[t]	5.59±0.32[r]	4.99±0.15[s]	4.89±0.10[q]
	F	3.2±0.67[a]	4.55±0.17[a]	5.83±0.34[b]	6.16±0.54[t]	5.88±0.45[r]	3.2±0.67[s]	4.55±0.17[q]
Insulin (pg/ml)	M	3022±595[a]	1888±150[a]	5958±731[by]	2565±443[x]	3312±633[x]	2052±406	1979±816
	F	1599±394[ab]	2375±1133[ab]	1692±513[az]	1880±274[z]	1321±343[z]	2460±141	1699±279
Amylin (pg/ml)	M	43.1±6.8[a]	418.6±145.2[b]	75.0±11.1[a]	56.4±10.5	63.0±5.7[r]	43.3±6.0	28.4±3.6[q]
	F	37.3±6.4[ab]	51.5±9.7[a]	52.2±8.1[ab]	52.8±9.4	54.3±4.8[r]	49.3±22.1	32.6±3.4[q]
Ghrelin (ng/ml)	M	266.8±52.1	171.4±32.1	233.1±39.7	159.4±36.8[s]	235.1±39.2	344.6±61.1[t]	306.2±56.5
	F	529.1±74.4	402.7±65.7	344.7±55.4	246.1±50.7[s]	369.6±47.7	454.0±77.1[t]	404.3±41.7
GIP (ng/ml)	M	49.9±6.3	87.3±14.0	49.9±6.3	46.5±8.3	47.8±6.6	52.3±8.7	62.9±8.6
	F	49.7±5.3	34.9±6.1	41.6±8.4	33.4±9.2	42.1±3.2	67.6±13.9	69.2±11.4
PYY (pg/ml)	M	72.4±6.3	54.0±10.9	64.1±4.4[x]	62.7±7.5[x]	262.5±43.2[y]	52.8±8.2	243.7±26.0
	F	62.2±13.6	36.1±4.9	55.8±5.0[x]	63.5±8.9[x]	201.3±35.1[y]	48.8±10.1	147.9±25.7
GLP-1 (pg/ml)	M	6.5±0.42[ab]	5.4±0.19[ab]	7.22±0.48[abx]	6.0±0.58[x]	8.4±0.79[y]	6.5±0.22	7.6±0.78
	F	6.4±0.37[b]	4.82±0.35[ab]	4.45±0.72[ax]	6.36±0.37[x]	8.54±1.5[y]	5.87±0.70	7.5±0.41
Leptin (ng/ml)	M	20.4±1.9	18.4±2.4	27.2±2.5[y]	21.0±4.2[y]	14.5±2.2[x]	14.2±1.8	9.3±1.2
	F	12.9±1.4	11.3±4.1	11.1±2.1[y]	10.3±1.6[y]	6.5±1.0[x]	10.7±1.6	9.3±2.1
HOMA-IR	M	15.3±2.9[a]	9.8±1.1[a]	35.9±4.8[by]	20.2±3.8[x]	19.0±3.4[qx]	10.9±2.2	11.0±3.9[r]

	F	8.8 ± 2.2^{abcd}	35.5 ± 4.5^{c}	7.1 ± 1.5^{dz}	10.4 ± 1.6^{z}	5.8 ± 1.1^{rz}	12.1 ± 6.3	7.8 ± 1.3^{rq}
Liver TG	M	33.1 ± 2.5	30.4 ± 4.0	43.1 ± 3.7^{y}	36.6 ± 3.1^{ty}	30.8 ± 3.1^{x}	29.5 ± 3.6^{st}	31.1 ± 3.3
	F	36.8 ± 2.0	35.5 ± 4.5	32.2 ± 1.6^{y}	26.8 ± 2.5^{sy}	23.2 ± 1.5^{x}	32.1 ± 2.1^{st}	27.8 ± 2.2

Values are means ± SEM, $n = 4 – 12$. Superscripts ^{a,b,c} indicate significant differences between R, H, and C1; ^{x,y,z} indicate significant differences between C1, HP1, HF1; ^{q,r} indicate significant differences between HF1 and RF; ^{s,t} indicate significant differences between HP1 and RP.

Table 5.7. Hepatic gene expression in the offspring of dams fed a control, high-protein or high-prebiotic fiber diet during pregnancy and lactation and then re-matched to their respective diets in adulthood

		Diet Group			*P* (2-way ANOVA)		
		C	HP1	HF1	Diet	Sex	Diet × Sex
SREBP1c	M	4.6±0.67	5.5±0.73	2.6±0.7	0.002	0.002	0.143
	F	1.7±0.53	4.0±0.66	2.1±0.47			
ACC	M	15.0±1.6	13.3±2.0	10.9±2.0	0.032	0.553	0.097
	F	12.6±1.3	18.9±3.1	10.6±1.2			
FAS	M	4.0±0.74	6.8±2.0	4.7±1.1	0.002	0.001	0.124
	F	8.2±0.92	18.2±3.1	13.0±1.9			
PGC1α	M	24.1± 5.8[a]	88.9±19.6[b]	45.9±9.8[a]	0.001	0.064	0.972
	F	45.7±6.4[a]	110.9±21.1[b]	62.5±13.6[a]			
Glucose-6-	M	17.1±2.7[ab]	23.6±5.5[a]	19.5±3.8[ab]	0.001	0.031	0.014
Phosphatase	F	20.5±2.3[a]	43.6±4.4[b]	16.7±2.9[a]			
AMPKα1	M	10.7±1.4	16.5±1.6	11.7±.9	0.006	0.587	0.121
	F	15.2±1.0	16.2±2.8	9.7±1.8			

Values are means ± SEM, *n* = 8 – 10 per group.

5.4 Discussion

Our first aim was to examine the effects of transient versus persistent exposure to a high fat, high sucrose diet in offspring whose mothers consumed a control diet. We compared a variety of metabolic outcomes in animals that were never exposed to HFS, exposed for the final 14 weeks and those that were re-matched to control diet following the 6 week HFS challenge. Perhaps not surprising given evidence that a diet high in fat and sugar will increase glycemia [249, 250], the animals that were never exposed to HFS diet had lower fasting glucose and glucose AUC compared to those that did consume the diet for 8 weeks. Females in the R group who were exposed solely to control diet pre- and postnatally also had higher fasting GLP-1 and GLP-1 AUC than those with exposure to the HFS diet. GLP-1 is an incretin hormone with glucose-lowering properties, secreted from the L-cells of the distal small intestine and proximal colon[251]. This hormone has been shown to be negatively impacted by high fat diets with obesity-prone rats consuming a high fat diet having lower plasma GLP-1 levels and a decreased number of L-cells in the distal small intestine[252], therefore increased GLP-1 levels in the R group would be expected. In contrast to what one might expect with prolonged exposure to a HFS diet [249, 250], animals that remained on HFS for the final 14 weeks of the study in fact had lower fasting glucose compared to the C1 animals in which the exposure to the HFS diet was transient. Insulin levels of animals switched from a cafeteria diet to chow have been reported to be three times higher than those never consuming a cafeteria diet[253]. Similarly, our animals re-matched to the control diet after 8 weeks of HFS consumption did have higher fasting insulin levels than R animals. However, contrary to results from South et al.[253], our male animals maintained on the HFS diet did not have significantly different fasting insulin levels than those never exposed to HFS. Our H animals had lower fasting glucose than C1 animals which may be in part due to the increased energy intake of C1. While neither is considered a low carbohydrate diet, the carbohydrate content of AIN-93M is higher than that of the HFS diet. Diets with lower carbohydrate content have been associated with improved measures of glycemic control, such as lower fasting plasma glucose[254]. HOMA-IR scores were consistent with the patterns of glycemia observed in the three groups of rats with greater insulin resistance

seen with transient exposure versus no exposure or prolonged exposure to HFS diet. Our results suggest the longer-term, lower carbohydrate diet, though high in fat, had more beneficial effects on glucose control than the control diet which had a high carbohydrate content.

Despite the differences observed in glucose tolerance, rats that were re-matched to control diet after the HFS challenge did not display any differences in body weight or body composition from rats that never consumed HFS, or those who continued to consume HFS over the final 6 week re-matching period. This could be considered "persistent" obesity, as has been reported elsewhere[255, 256]. Interestingly, other studies have reported weight loss following a switch from a high fat diet to a control diet that was a chow formulation[257, 258]. Whether the persistence of obesity occurred in our animals is difficult to conclude due to the similar body weight and composition to those rats never exposed to the HFS diet. The control diet used in this study was AIN-93. Unpublished data from our laboratory has shown that percent body fat is higher in rats consuming the AIN-93 diet compared to a standard chow diet and is not statistically different from fat mass in rats fed a HFS diet (Neustadter and Reimer, unpublished results). Rodent diet in a powdered form as opposed to pellet form is easier for animals to eat and typically contains ingredients that make it more palatable, such as dyetrose. These factors could easily result in increased food intake with powdered versus pelleted diets. In a comparison of soft and hard food, Sako et al.[259] found that when given the choice, rats would consume more soft food than hard pellets. The lack of weight loss in our C1 animals compared to other studies that switch from an HFS to control diet[257, 258] could be due to the palatability and softness of our diet which likely impacted food intake and subsequent body weight. Indeed reference animals consumed more energy during the final food intake measurement period than C1 animals, neither of which was different from H animals.

South et al.[253] reported persistent hypophagia after switching rats from a cafeteria diet to a chow diet. Conversely, our rats exhibited hyperphagia following the switch from HFS to control diet given that they consumed more energy than H animals after the switch, although this was transient and no longer evident three weeks later. Maintenance

of a steady weight after switching from a high energy diet to chow has also been observed before, with decreased food intake and weight loss occurring only when there was a drastic difference in palatability of the diet from liquid Ensure to chow[260].

Our second aim was to determine if the effects of a transient HFS diet could be mitigated to a greater extent by re-matching to a HF or HP diet following the HFS challenge. HF1 animals had decreased weight and percent body fat compared to both C1 and HP1. We previously reported that a maternal diet high in prebiotic fiber provided protection to females against diet-induced obesity, and a predisposition to diet-induced obesity for female HP1 offspring[221]. Rats fed HF diet from weaning to adulthood also demonstrated a resistance to obesity compared to control and HP-fed animals[117]. After re-matching to the weaning diet, Reimer et al.[117] showed that HP animals had higher body weight than C and HF animals, partially due to increased food intake. In the current study, the decreased body weight and adiposity is likely in part due to the increased levels of the satiety hormones GLP-1 and PYY. These hormones have frequently been reported to decrease food intake and increase feelings of fullness[261].

HOMA-IR scores were higher in C1 than HF1 and HP1 males. This reflects lower insulin resistance in HP1 and HF1 males versus C1, while females did not differ across diets. We found that after HFS feeding HP1 animals had improved HOMA-IR scores compared to C1 and this either persisted through the re-matching period or was assisted by the lower carbohydrate diet. The HF diet was able to reverse some of the detrimental effects of the HFS feeding in this regard, as immediately after HF1 animals did not differ from C1[221].

HF1 animals had decreased liver triglyceride concentration compared to HP1, retaining the same pattern as previously reported following the HFS challenge[221]. HP1 animals in addition to having greater hepatic triglyceride levels also demonstrated increased gene expression of the lipogenic enzymes acetyl Co-A carboxylase (ACC), fatty acid synthase (FAS) and sterol regulatory element-binding protein-1c (SREBP1c) in the liver. These enzymes are integrally involved with *de novo* fat synthesis and have been shown to increase with increasing carbohydrate content of the diet[262]. A high protein diet consumed in the context of energy restriction has been shown to improve fatty liver

in sucrose-fed obese rats[263]. In our study, despite the high protein content of the HP diet, a considerable percentage of calories (44.67%) still came from carbohydrate. Combined with the relative hyperphagia exhibited, particularly by male HP1 animals immediately after being re-matched to their maternal diet, weight loss did not occur and liver triglyceride content remained higher than HF1 animals. C1 animals also had higher liver triglyceride levels than HF1 animals and again this was likely related to the high carbohydrate content of the diet (63.8% of calories from carbohydrate), combined with a lack of caloric restriction or weight loss during the 6 week re-matching period.

Our final aim was to compare the effects of the high protein and high prebiotic fiber diets after an HFS challenge in animals with maternal exposure to the diet versus those that had no previous exposure. Re-matching to maternal diets had both beneficial and negative consequences with differences in intestinal characteristics, body composition and glycemia.

HP1 animals had increased fasting plasma glucose levels compared to “naïve” RP animals. A high protein diet has previously been shown to improve glycemia when used in diet interventions[264, 265]. While not significant there was also an observable increased weight loss in RP versus HP1 animals that could play a role in reduced fasting glucose levels, as weight loss can significantly improve glucose control[266]. Male HP1 animals also consumed more of their respective diets immediately after re-matching than those animals that had no previous exposure to the diet. While at 3 weeks of age all animals were weaned onto AIN-93G, at 2 weeks of age pups will often start to sample their mother’s food. This early, though brief, exposure could program a preference, or “taste”, for these diets later in life. In rats fed a high fat diet early in life, a preference for that high fat diet was observed when given a choice between 3 different diets that was greater than the preference observed in animals without previous exposure to the diet[267].

In RF the magnitude of weight loss was much greater in response to the fiber diet than in HF1 animals, and these animals also had decreased fasting glucose compared to HF1. Given evidence that weight loss significantly improves glucose control[266], this could account for the lower blood glucose in RF animals despite having a higher body

weight at study termination. Cecum weight was also increased in RF animals. It has previously been shown that supplementation of fructooligosaccharide results in enlargement of the cecum[268]. As the cecum is the location for fermentation of indigestible components of the diet, increased fermentation will increase size of the cecum[269]. In HF1 versus HP1 and C1 animals there was also an increase in cecum size. One of the bacterial species increased with prebiotic fiber intake is *Bifidobacterium*. This species is strongly associated with gut health and has also been found to have an impact on whole body metabolic health[243]. Specifically, increases in *Bifidobacterium* are associated with improved measures of glycemia and insulinemia[25]. Examination of the gut microbiota in the context of mismatching and re-matching could provide additional insight into metabolic differences triggered by our mismatched and re-matched diets.

Changes in diet composition across the lifespan are a common occurrence[270, 271]. Evidence suggests that when the dietary mismatch across periods is great, the susceptibility to chronic disease increases[37]. This work suggests that re-matching to a maternal diet high in prebiotic fiber has clear benefits for reducing the negative impact of a transient HFS diet, including reduced body weight and body fat and improved satiety hormone response. Conversely, detrimental effects on body composition and hepatic lipogenic gene expression were observed when rats were re-matched to a HP diet. The surprising lack of difference in body fat between C1, R and H animals suggests that the obesogenic potential of the AIN-93 diet warrants further investigation.

Chapter Six: Long-term intake of a high prebiotic fiber diet by offspring exposed to gestational protein restriction has differential effects on glucose tolerance and intestinal permeability in rats[6] [7]

6.1 Introduction

The influence of maternal dietary, metabolic and environmental factors on offspring health have been well documented in animal models and increasingly so in humans[272]. While the maternal stressors that lead to aberrant offspring health are numerous, one stressor, intra-uterine growth restriction (IUGR) has been shown to lead to early effects such as perinatal mortality[273] and necrotizing enterocolitis[274]. The effects of IUGR, however, also persist and increased risk of metabolic syndrome, cardiovascular disease, as well as an increased susceptibility to irritable bowel syndrome can occur later in life[275-278]. Intra-uterine growth restriction is frequently modelled in animals using a protein restricted (PR) diet during pregnancy, lactation, or both periods. Offspring exposed to the nutritional deficiency display changes in the development of the insulin axis[279], hypothalamic nuclei[73], and numerous organs[4], as well as changes in body composition, activity levels and food intake[280] and gut microbiota[281]. One organ that is remarkably impacted by maternal protein restriction is the gastrointestinal tract. Both the colon[282] and small intestine display deficits in intestinal length, DNA content, number and height of intestinal villi and decreased migration of the enterocytes along the intestinal villi in response to *in utero* protein restriction [90].

Thus far, there is modest evidence that the effects of a maternal PR diet can be reversed in the offspring. Prenatally, supplementing a maternal protein-restricted diet with folic acid or taurine during pregnancy can prevent some of the adverse effects of protein restriction in the offspring[283-285]. Postnatally, Vickers et al.[36] found that leptin injections in female pups of undernourished dams normalized high-fat diet induced

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[6] A version of this paper has been submitted to FASEB Journal.
[7] A portion of this work has been presented in part at Experimental Biology 2013. *Prebiotic fiber does not improve offspring `leaky gut`* from maternal low protein diet. FASEB J *27*(Meeting abstracts): 1058.1.

weight gain, adiposity, food intake, plasma leptin, insulin and locomotor activity to that of offspring from adequately nourished dams. Dietary interventions in the postnatal period also hold promise given that plasticity, or the ability to respond to the environment, has been demonstrated in response to folic acid supplementation at 4 weeks of age[49]; and a weaning diet supplemented with probiotics, prebiotics and long chain poly-unsaturated fatty acids improved small intestine permeability and growth after maternal separation-induced stress in rats[286].

Prebiotic fiber has been shown to have positive effects on numerous aspects of health including enhanced weight and body fat loss, improved glucose control and increased bifidobacteria in the gut[117, 118]. Beneficial effects on intestinal growth and function have also been observed[112, 119, 287]. Given its documented effects, prebiotic fiber has the potential to address a number of components of the adverse programming found in the offspring of dams fed a protein restricted diet during pregnancy. As such, we set out to determine if a weaning diet high in prebiotic fiber would improve gut development, gut microbiota and glucose and lipid metabolism in offspring exposed to maternal protein restriction during pregnancy.

6.2 Methods

6.2.1 Ethical Approval

The University of Calgary Animal Care Committee approved the experimental protocol which was conducted in accordance with the Guide for the Care and Use of Laboratory Animals.

6.2.2 Animals and Diets

Twenty-seven virgin Wistar dams were obtained from Charles River (Montreal, QC, Canada) and housed in a temperature and humidity controlled facility with a 12-hour light/dark cycle. After 7 days of acclimatization, animals were randomized to a protein-restricted diet (PR) (8% wt/wt) or control diet (C) (AIN-93G, 20% wt/wt). Diets were

purchased from Dyets Inc. (Bethlehem, PA, USA). Diet composition can be found in Tables 6.1 and 6.2. Dams consumed the diets for one week prior to being bred with male Wistar rats in wire-bottomed cages. Following the identification of a copulation plug, dams were housed individually and continued to consume their assigned experimental diet (PR or C) *ad libitum* until parturition. At parturition all dams consumed AIN-93G. Dams were weighed weekly and food intake was measured throughout week 2 of pregnancy, as well as during the first week post-partum.

6.2.3 Body composition, glucose tolerance and tissue sampling

On the day after birth, pups were weighed and litters were culled to 10 pups, 5 males and 5 females where possible. Offspring were weighed weekly for the remainder of the study. Food intake was also measured for 5 consecutive days at weeks 5, 9, 15 and 19 by subtracting the weight of the cup and diet from the previous day's weight. At weaning (21d), 1 male and 1 female pup were randomly selected from each litter to be weaned onto a control diet (AIN-93G; 20% protein wt/wt) and 1 male and 1 female pup were randomly selected to be weaned onto a high prebiotic fiber diet (HF; 21% wt/wt, 1:1 ratio oligofructose and inulin). At 10 weeks of age, offspring were switched to the maintenance versions of their diets (14% protein wt/wt) and HF animals were switched to a diet of 10% (wt/wt) prebiotic fiber (Table 6.1). Pups not selected for inclusion in the study underwent a DXA scan (Hologic ODR 4500; Hologic Inc., Bedford, MA, USA) while lightly anaesthetised with isoflurane at one week post-weaning. Hologic QDR software for small animals was used to determine lean and fat mass. At 23 wk of age, rats underwent an oral glucose tolerance test (OGTT). Following an overnight fast, blood was sampled from the tip of the tail in conscious rats followed by an oral glucose gavage (2 g/kg). At 15, 30, 60, and 90 min post-glucose gavage, additional blood samples were collected for satiety hormone analysis. Blood glucose concentrations were determined immediately at each time point with a blood glucose meter (Accu-Chek Blood Glucose Meter, Laval, QC, Canada). Blood was collected in tubes containing diprotinin-A (0.034 mg/ml blood; MP Biomedicals, Irvine, CA, USA); Sigma protease inhibitor (1 mg/ml blood; Sigma Aldrich, Oakville, ON, Canada) and Roche Pefabloc (1mg/ml of blood;

Roche, Mississauga, ON, Canada) and then centrifuged at 1600×g for 12 min at 4ºC. Plasma was stored at -80ºC until analysis. One week after the OGTT and one day prior to study termination, rats underwent a DXA scan (as above) to determine body composition. At study termination, rats were fasted overnight and then anaesthetized using isoflurane. A blood sample was collected from the hepatic portal vein for analysis of GLP-2 and lipopolysaccharide (LPS). Rats were killed via over-anaesthetisation and aortic cut. The liver, stomach, small intestine, cecum, and colon were weighed, snap frozen in liquid nitrogen and stored at -80ºC.

6.2.4 Plasma Analysis

A Milliplex Rat Gut Hormone kit (Millipore, St. Charles, MO, USA) and Luminex instrument were used to measure ghrelin (active), insulin, amylin (active), leptin, glucose-dependent insulinotropic polypeptide (GIP) (total), GLP-1 (active) and peptide tyrosine tyrosine (PYY) (total). Homeostasis model assessment of insulin resistance (HOMA-IR) was calculated from fasting insulin and fasting glucose. An ELISA was used to measure fasting concentrations of active GLP-1 and GLP-2 (Millipore, St. Charles, MO, USA). Fasting LPS was measured using a PyroGene Recombinant Factor C Endotoxin Endpoint Fluorescent Detection assay (Lonza Group Ltd., Basel, Switzerland) according to manufacturer directions.

6.2.5 Hepatic Triglyceride Analysis

Triglyceride content of the liver was quantified using 25mg of tissue according to the manufacturer guidelines of the GPO reagent set (Pointe Scientific Inc., Lincoln Park, MI, USA).

6.2.6 RNA Extraction and Real-Time PCR

Total RNA was extracted from the colon using QIAzol reagent (Qiagen, Germantown, MD, USA). Reverse transcription was performed with an input of 1 μg of

total RNA using the 1st strand cDNA synthesis kit for RT-PCR (Invitrogen, Carlsbad, CA, USA) with oligo d(T)15 as a primer. The cDNA was amplified using primers synthesized by the University of Calgary Core DNA Services (Calgary, AB, Canada) and analyzed by real time PCR. Primer sequences were according to our previous work[112]. A melt curve showed the melting point of the PCR product of interest. mRNA levels of sodium-glucose transporter-1 (F: CTACATCCAGTCCATCACCAGTTAC, R: CCAATCAGGAAGCCGAGAATCAG), tight junction protein-1 (F: CCATGCCTCCTCCTCCTC, R: ACGGAATTGCCTTCACTCTG), mucin-2 (F: CACCATTACCACCACCTCAG, R: CGATCACCACCATTGCCATTG and trefoil factor-3 (F: GTCCTGGTTGCTGGGTCCTC, R: CCACGGTTGTTACACTGCTCTG) were measured in the colon. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was verified as a suitable housekeeping gene for the tissues of interest and GAPDH primers included as an internal control in the reactions. The $2^{-\Delta CT}$ method [ΔCT = CT (gene of interest) – CT (reference gene)] was utilized for the data analysis where threshold cycle (CT) indicates the fractional cycle number at which the amount of amplified target reaches a fixed threshold[189]. The ΔCT is the difference in threshold cycles for the gene of interest and GAPDH.

6.2.7 Fecal DNA Extraction and Real-Time PCR

Fecal pellets were collected from the dams at parturition and offspring cecal contents were collected at study termination. Total bacterial DNA was extracted from ~200 mg of fecal or cecal contents using FastDNA Spin Kit for Feces (MP Biomedicals, Lachine, QC, Canada) and quantified using PicoGreen DNA quantification kit (Invitrogen, Carlsbad, CA, USA). All samples were brought to a concentration of 4 ng/µl prior to storage at -20°C for later analysis. Amplification and detection were conducted in 96-well plates with SYBR Green 2 × qPCR Master Mix (BioRad Laboratories Inc., Hercules, CA, USA). Samples were run in duplicate with a final volume of 25 µl containing 0.3 µM primer and 20 ng template gDNA. qPCR was performed according to previously published protocol [288] with the group specific primers provided in Table 4.3. The specificity of the primers and the limit of detection were determined according

to Louie et al.[222]. The 16S rRNA gene copies value was calculated according the following webpage: http://cels.uri.edu/gsc/cndna.html using average genome sizes. Standard curves were normalized to the copy number of the 16S rRNA gene obtained from the following webpage: http://rrndb.mmg.msu.edu/index.php.

6.2.8 Statistical Analysis

All data are presented as mean ± SEM. Data collected from the dams was analyzed using a one-way ANOVA. In offspring a three-way ANOVA with factors of maternal diet, offspring diet and sex was run and a Bonferroni correction was applied such that $P \leq 0.05$ was considered significant. Statistical analysis was performed using SPSS v 19.0 software (IBM, Chicago, IL, USA).

Table 6.1. Diet compositions

Ingredient	Control (growth)	Protein Restricted	Control (maintenance)	10% Prebiotic Fiber
Cornstarch (g/kg)	397.5	647.7	465.7	365.7
Casein (g/kg)	200.0	83.6	140.0	140.0
Dyetrose (g/kg)	132.0	-	155.0	155.0
Sucrose (g/kg)	100.0	100.0	100.0	100.0
Soybean Oil (g/kg)	70.0	70.0	40.0	40.0
Alphacel (g/kg)	50.0	50.0	50.0	50.0
AIN-93M Mineral Mix (g/kg)	35.0	35.0	35.0	35.0
AIN-93-VX Vitamin Mix (g/kg)	10.0	10.0	10.0	10.0
L-Cystine (g/kg)	3.0	1.2	1.8	1.8
Choline bitartrate (g/kg)	2.5	2.5	2.5	2.5
Inulin (g/kg)	-	-	-	50.0
Oligofructose (g/kg)	-	-	-	50.0

Table 6.2. Macronutrient composition

% of Total Calories	Control (growth)	Protein Restricted	Control (maintenance)	10% Prebiotic Fiber
Carbohydrate	63.80	73.31	73.98	74.41
Protein	19.45	8.13	14.11	13.87
Fat	16.74	16.75	9.99	9.82

Figure 6.1. Experimental design

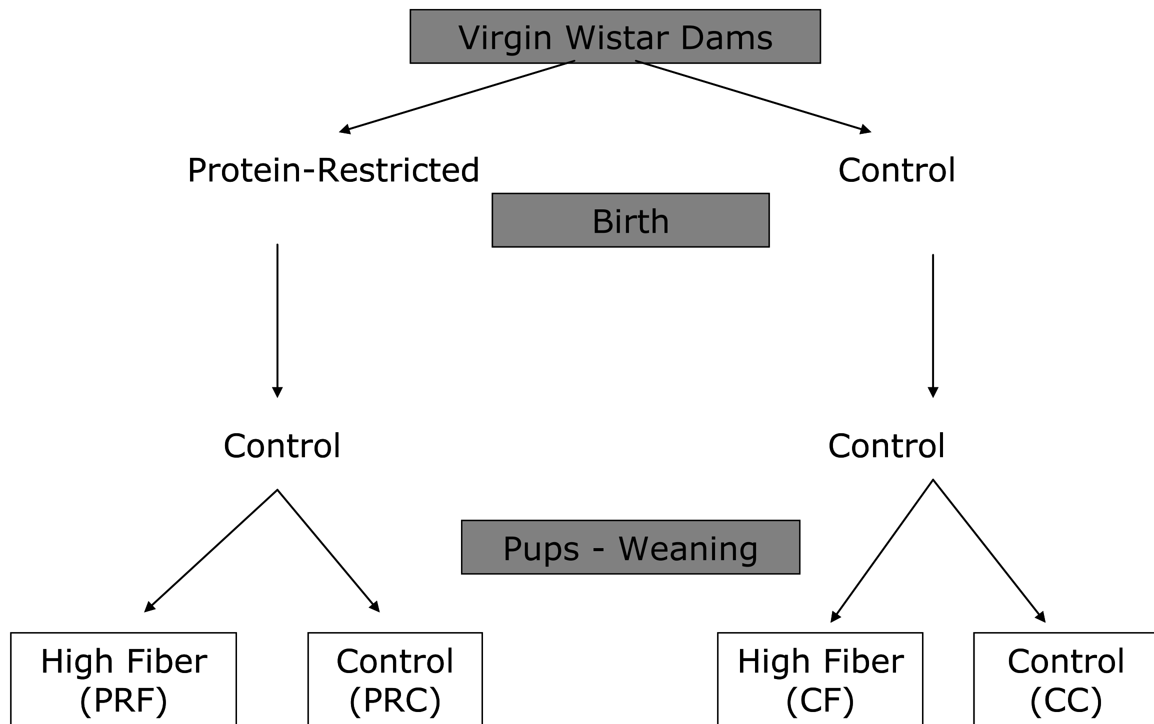

Figure 6.1: Schematic of the experimental design.

6.3 Results

6.3.1 Dams and Litters

Body weight in the dams did not differ at any point in the study except during the third week of pregnancy when PR dams gained significantly less weight than control dams (P<0.001) (Table 6.3). There was a trend for an increased number of still births from PR dams (C: 0, PR: 0.44±0.19, P=0.064) but there was no difference in the number of live births or the number of males or females born to PR and C dams. During the second week of pregnancy, PR dams consumed significantly more food than controls (P=0.032), eating 15% more, thereby increasing their caloric intake by almost 18%. There were no differences in food intake in the dams during the first two weeks of lactation.

6.3.2 Offspring Growth and Food Intake

Pups from the PR dams had lower birth weight compared to C dams (P=0.004) and male pups weighed more than female pups for both diet groups (P<0.001). During the remainder of the suckling period there were no significant differences in pup weight. The DXA scan of pups not selected to continue in the study showed that lean mass was lower (P=0.036) and percent body fat higher in offspring from PR dams versus C (P=0.007) (Table 6.3). Growth trajectories of the pups are shown in Figure 6.2A.

Throughout the study a number of animals died or had to be euthanized due to sickly appearance and/or weight loss, although the facility veterinarian was unable to determine a cause of death or illness. Two male offspring from the control dam/fiber fed offspring group (CF) died at 7 and 8 weeks of age, while 3 more were euthanized between weeks 7 and 9. Four females from the CF group were euthanized between weeks 7 and 9. A total of three males from the low-protein dam/fiber fed offspring group (PRF) died and one was euthanized between weeks 7 to 9 and 1 female died at week 6.

Food intake in offspring was significantly affected by the interaction of time, offspring diet, sex and maternal diet ($P<0.02$) (Figure 6.2B). Energy intake increased with age, although to varying degrees based on sex and offspring diet. Postnatal consumption of fiber by offspring of C dams reduced energy intake compared to control at 5 and 9 weeks in males. Postnatal consumption of fiber by offspring of PR dams reduced energy intake compared to control in males at 19 weeks and in females at 9 weeks ($P<0.05$). At 9 and 15 weeks, PRF males consumed more than CF males.

At study termination, the interaction between diet and sex affected body weight ($P=0.02$), with males weighing more than females, and males consuming control diet having a higher body weight than those consuming fiber (Table 6.4). The interaction between offspring diet and sex also affected fat mass, lean mass and length, all of which were greater in male than female offspring. Fat mass was greater in males consuming C than F ($P=0.046$), lean mass was greater in females consuming F than C ($P=0.048$) and naso-anal length was greater in males consuming C than F ($P=0.007$).

The high fiber diet increased small intestine length and cecum weight compared to control ($P<0.001$). The interaction of maternal diet and sex affected liver weight, with males from C dams having higher liver weight than males from PR dams ($P=0.034$). The interaction between offspring diet, sex and maternal diet influenced the weight, length and weight per length of the colon in offspring ($P<0.01$) (Table 6.4). The weight of the colon was lower for male PRF than CF while in females PRF had higher colon weight than CF. Colon length was greater in females consuming F versus C, while in males this was only true for PR offspring.

Table 6.3. Weight gain and litter statistics of dams fed a control or protein restricted diet during pregnancy

	Control	Protein Restricted
Dam preconception weight	304.0±4.0	303.6±1.6
Dam weight gain pregnancy week 1 (g)	49.5±2.2	60.6±6.2
Dam weight gain pregnancy week 2 (g)	41.6±1.2	45.7±3.4
Dam weight gain pregnancy week 3 (g)	77.0±1.3	62.2±3.2*
Dam energy intake (kcal/d)	93.7±2.7*	110.4±5.5
Dam food intake (g/d)	26.0±0.7*	29.9±1.4
Female pup birth weight (g)	5.88±0.08	5.63±0.08*
Male pup birth weight (g)	6.17±0.08	5.8±0.08*
# Pups	15.75±0.73	14.5±0.58
# Males	7.8±0.62	7.4±0.26
# Females	7.7±0.33	6.9±0.69
Pup lean mass at 4 weeks (g)	66.0±1.9	59.9±2.1*
Pup body fat at 4 weeks (%)	21.7±0.45*	23.5±0.48

Values are mean ± SEM with n=13 per group**.** The superscripts * indicates significant differences between diets ($P<0.05$). Food and energy intake was measured during the second week of pregnancy.

Table 6.4. Physical characteristics of offspring derived from control or protein-restricted dams at 24 weeks of age

	Sex	Treatment Group				3-way ANOVA P-values						
		CC	CF	PRC	PRF	Diet	Sex	MatDiet	D*S	D*M	S*M	D*S*M
Body Mass (g)	M	707.1±21.9	649.5±28.2	719.7±20.9	620.1±26.1	0.018	0.001	0.891	0.020	0.766	0.516	0.326
	F	383.1±20.8	371.3±24.4	384.7±20.0	395.4±20.8							
Body Fat (%)	M	31.2±2.3	23.2±3.2	34.7±2.3	28.4±2.9	0.001	0.624	0.403	0.729	0.404	0.127	0.713
	F	33.1±2.3	35.1±2.8	29.6±2.2	26.0±2.4							
Fat mass (g)	M	216.7±17.8	151.7±23.0	255.4±17.0	164.4±23.0	0.001	0.001	0.376	0.046	0.951	0.313	0.307
	F	131.8±17.0	93.7±19.9	115.4±16.2	106.6±17.0							
Lean mass (g)	M	490.7±9.4	497.8±12.8	464.3±9.4	437.5±11.8	0.505	0.001	0.032	0.048	0.247	0.001	0.255
	F	257.8±9.0	277.6±11.1	269.3±9.0	288.8±9.4							
Nasoanal Length (cm)	M	28.3±0.3	27.8±0.3	27.0±0.2	26.5±0.3	0.053	0.001	0.024	0.007	0.161	0.042	0.331
	F	23.2±0.2	23.4±0.3	23.2±0.2	23.3±0.3							
Liver (g)	M	19.7±0.7	18.8±0.9	17.8±0.7	16.9±0.9	0.522	0.001	0.201	0.315	0.934	0.034	0.937
	F	10.2±0.7	10.3±0.8	10.6±0.7	10.9±0.7							
Colon (g)	M	1.47±0.06^{ab}	2.10±0.08^{b}	1.50±0.05^{ab}	1.70±0.08^{a}	0.001	0.001	0.413	0.090	0.130	0.007	0.005
	F	1.13±0.05^{ab}	1.30±0.07^{a}	1.15±0.05^{ab}	1.45±0.06^{b}							
Colon (cm)	M	21.6±0.44^{ab}	21.0±0.85^{ab}	20.0±0.43^{a}	23.5±0.60^{b}	0.001	0.015	0.407	0.715	0.034	0.783	0.002
	F	19.4±0.47^{a}	21.5±0.52^{b}	20.0±0.47^{ab}	21.3±0.49^{ab}							
Colon (g/cm)	M	0.07±0.003^{ac}	0.10±0.006^{b}	0.08±0.002^{abd}	0.07±0.003^{a}	0.001	0.001	0.267	0.076	0.003	0.004	0.001
	F	0.06±0.003^{abcd}	0.06±0.003^{abcd}	0.06±0.003^{abcd}	0.07±0.003^{abcd}							

HOMA-	M	13.3±4.1	14.0±6.0	28.0±4.3	5.2±5.1	0.292	0.184	0.550	0.024	0.289	0.764	0.015
IR	F	10.5±4.3	9.9±4.8	6.8±4.1	15.6±4.3							

Values are mean ± SEM with n=6-10 per group. Mean values without a common superscript are significantly different (*P*<0.05). D, diet; S, sex; M, maternal diet

Figure 6.2. Longitudinal body weight

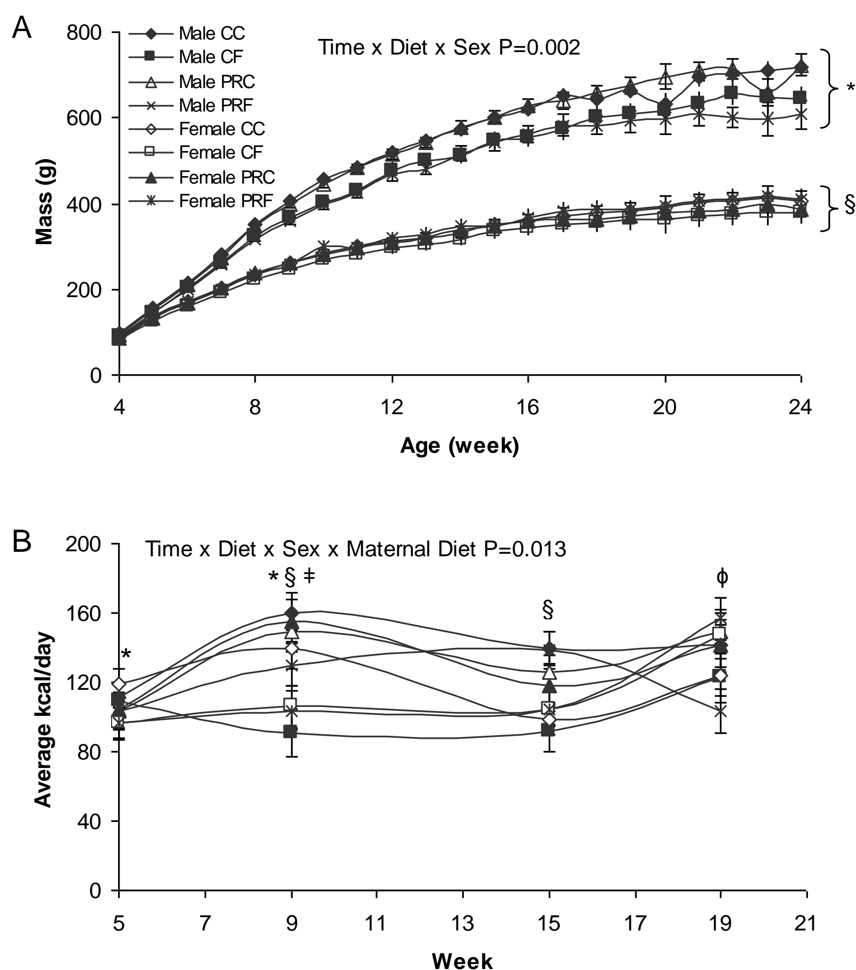

Figure 6.2. (A) Longitudinal body weight of males and females. Males had significantly greater body weight from week 4 to 24 (study termination). * indicates significantly higher body weight for males consuming control versus HF at weeks 5-22 and at week 24. § indicates significantly higher body weight for females consuming control versus HF at week 4. ($P<0.01$) (B) Male and female food intake at the four measurement periods. Data represent means ± SEM from 8-12 animals/group.

6.3.3 Gut Microbiota

Maternal diet had an effect on the gut microbiota profile in the dams at parturition. Both *C. leptum* (C: 46918.7±11527.1; PR: 81495.6±12089.8; P=0.05) and *Roseburia* (C: 695980.4±213946.6; PR: 8319000.8±2054736.9; P=0.003) were increased in PR dams.

In the offspring, *Roseburia* was significantly affected by the interaction of maternal diet, offspring diet and sex (P=0.036), with female CF being higher than PRF (Figure 6.2). The interaction between offspring diet and sex influenced *Lactobacillus, Bifidobacterium*, *Enterobacteriaceae* and Clostridiale Cluster I (P<0.05). *Lactobacillus* was higher in males consuming C versus F. *Bifidobacterium* was greater in offspring consuming F versus C. *Enterobacteriaceae* was greater in females consuming F than C, while Clostridiale Cluster I was greater in offspring consuming C than F and greater in control males than females. The interaction between maternal diet and offspring diet affected *C. leptum* and *Bifidobacterium* with offspring consuming control diet from control dams having greater abundance than those from PR dams for *C. leptum* and PRF offspring having greater abundance of *Bifidobacterium* (Figure 6.3A) than CF.

Independently, maternal diet, offspring diet and sex all influenced *C. coccoides* wherein PR offspring were higher than C offspring, those consuming C diet were higher than F and females were higher than males (Figure 6.2). Diet also affected *Bacteroides* wherein offspring consuming fiber had greater abundance than those consuming control, and for Clostridiale Cluster XI the reverse was true.

6.3.4 Plasma Satiety Hormones and Blood Glucose

The interaction between time, offspring diet and maternal diet affected insulin levels (P=0.05) (Figure 6.5). Throughout the OGTT, PRC offspring had higher insulin levels than PRF offspring except at 15 min. CF offspring had higher insulin levels than PRF offspring throughout the OGTT except at 15 min. The interaction of time and offspring diet affected PYY levels with animals consuming fiber having higher plasma

levels than controls at all time points (P=0.001). The interaction of time and sex affected glucose levels with males having higher glucose values at 90 min compared to females (P=0.001).

The interaction between offspring diet, sex and maternal diet influenced area under the curve (AUC) for insulin (P=0.001) (Figure 6.5), HOMA-IR (P=0.015) (Table 6.4) and plasma LPS (P=0.046) (Figure 6.4B). Insulin AUC was greater in male PRC than PRF. Male PRC had higher insulin AUC than CC and male CF had higher levels than PRF. Female CC offspring had higher insulin AUC than PRC. HOMA-IR scores were higher in male PRC than PRF and CC (P<0.05). Plasma LPS was lower in female CF than CC but in PRF females it was higher than PRC.

Maternal diet and sex had a significant effect on ghrelin and GIP AUC (P=0.038 and 0.014, respectively)(Figure 6.5). Ghrelin was higher in females consuming control diet than males. GIP AUC was higher in male offspring from PR dams than C dams. The interaction of offspring diet and maternal diet influenced amylin AUC (P=0.027) with PRF having greater amylin AUC than CF. Offspring diet alone had an effect on PYY AUC (P<0.001) and fasting GLP-2 levels (P=0.014). PYY AUC was greater in animals consuming F than C, as was fasting GLP-2 (P<0.05) (Figure 6.4C).

6.3.5 Gene Expression

The interaction between offspring diet, sex and maternal diet effected TJP1 mRNA levels in the colon (P=0.032) (Figure 6.3D). TJP1 expression was greater in male PRC than PRF and in female CC than CF. In females, CC had greater TJP1 expression than PRC, while CF had greater expression than PRF. Females from PR dams had decreased expression of SGLT1 in the colon compared to controls. MUC2 gene expression was affected by the interaction of maternal diet and sex (P=0.015) with females from C dams having higher mRNA levels than females from PR dams (Male C: 13.6±2.3, PR: 17.2±1.4; Female C: 15.2±3.1, PR:11.1±1.3). Maternal diet affected Tff3 expression (P=0.017) with offspring of C dams having higher mRNA levels than offspring from PR dams (C: 6.4±0.4, PR: 5.0±0.3).

Figure 6.3. Offspring gut microbiota

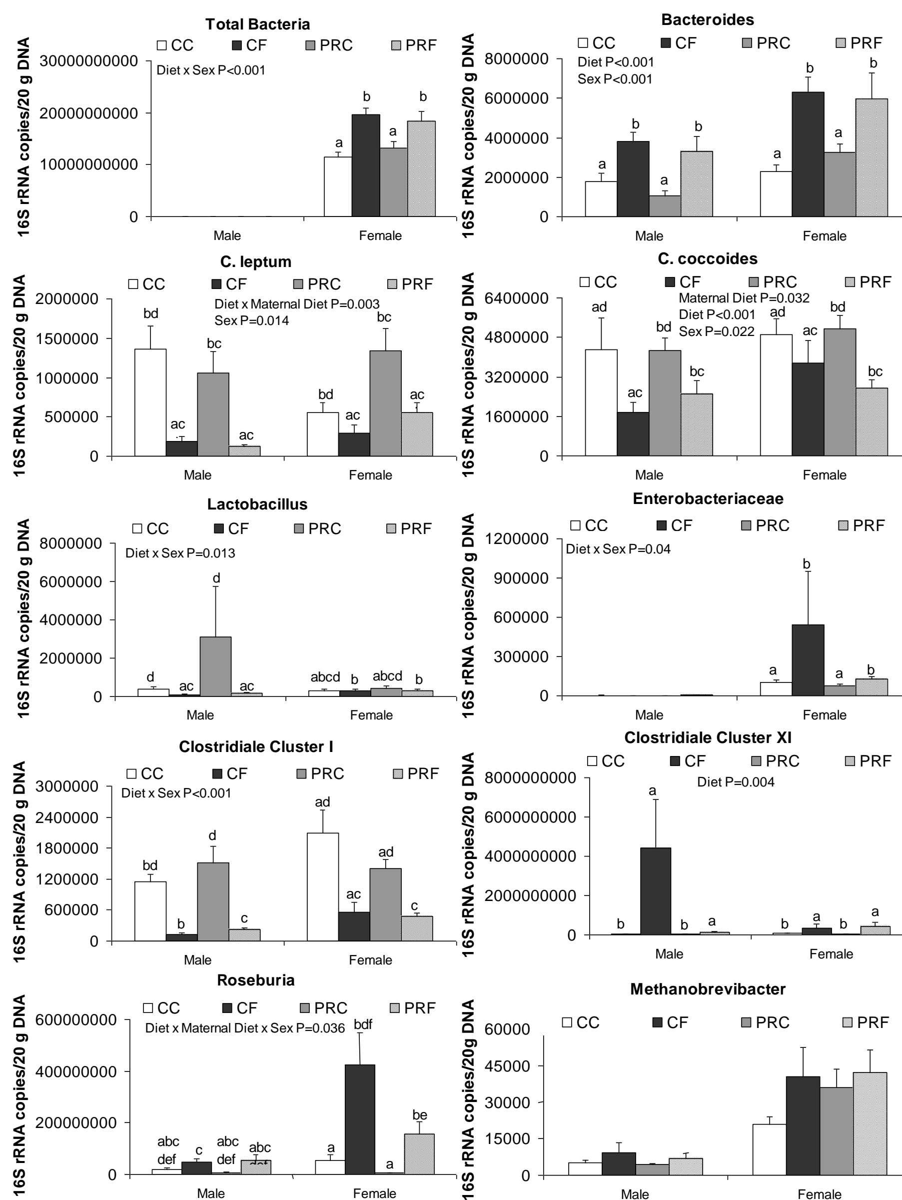

Figure 6.2. qPCR analysis of the offspring cecal gut microbiota. Data represent means ± SEM from 8-12 animals/group expressed as 16S rRNA gene copies/20ng total genomic DNA. CC, control dams/control offspring; CF, control dams/fiber offspring; PRC, protein restricted dams/control offspring; PRF, protein restricted dams/fiber offspring.

Figure 6.4. Gut-related metabolic health

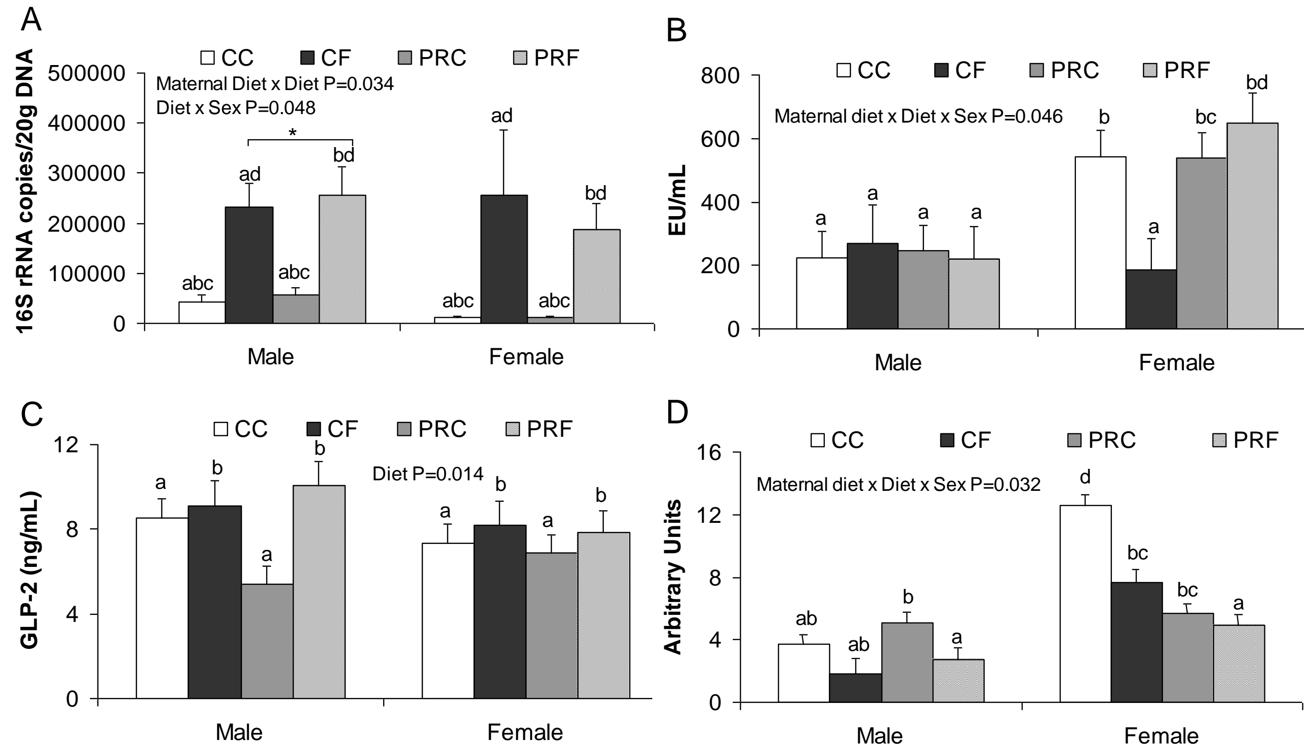

Figure 6.4. (A) *Bifidobacterium* in cecal contents; (B) fasting plasma levels of LPS; (C) mRNA levels of tight junction protein-1 (TJP1) in the colon and (D) fasting plasma levels of GLP-2 at study termination. In panel A, the * indicates males consuming HF had increased numbers over females consuming HF. In all panels, mean values without a common superscript are significantly different between the diets. Data represent means ± SEM from 8-12 animals/group.

Figure 6.5. Area under the curve for plasma measures during an OGTT

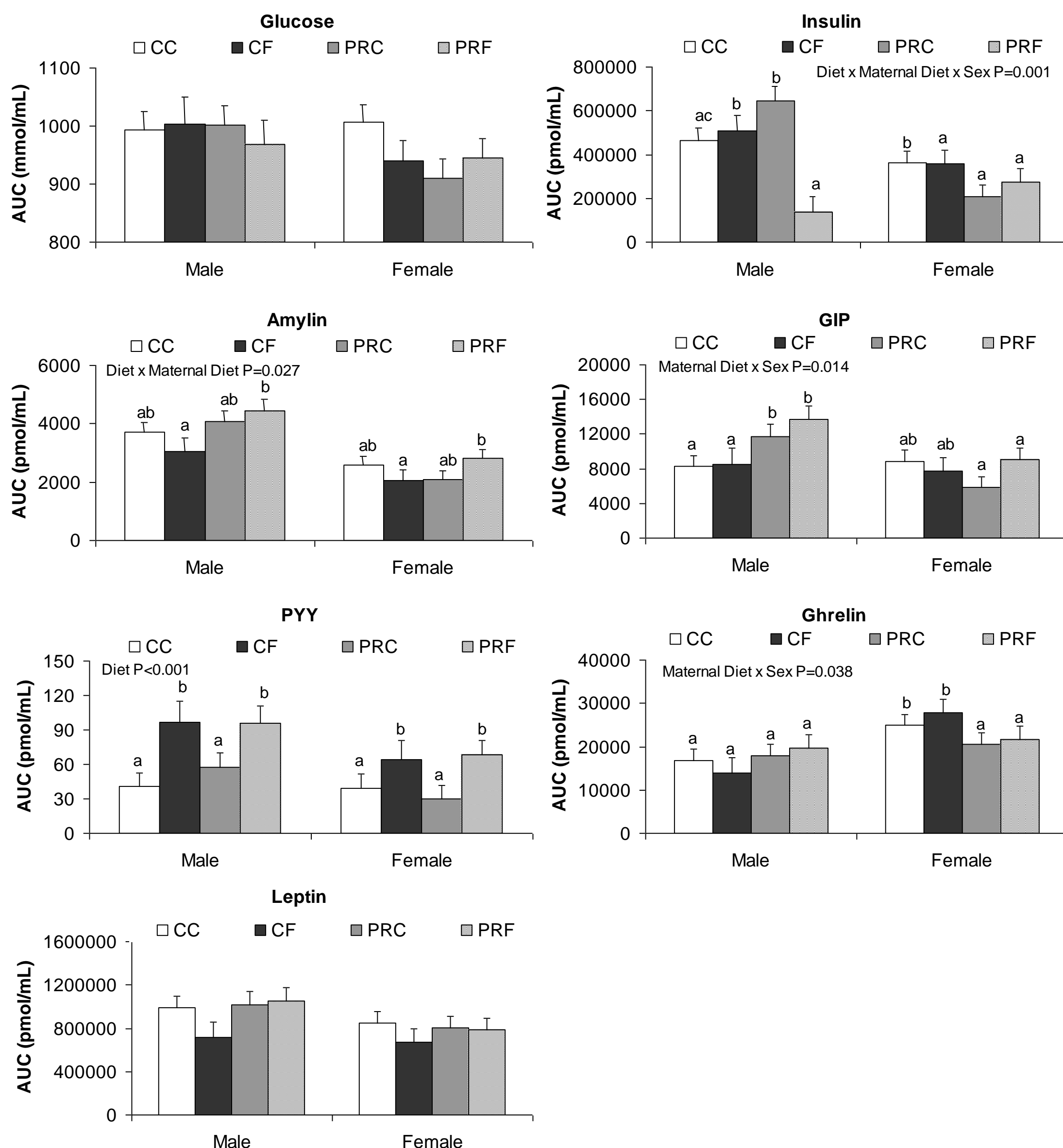

Figure 6.5. Area under the curve for plasma hormones and blood glucose. For insulin, values without a common superscript are significantly different between the diets within a sex. For all other hormones, mean values without a common superscript are significantly different between the diets. Data represent means ± SEM from 8-12 animals/group.

6.4 Discussion

Evidence from experimental and epidemiological studies demonstrates that exposure to aberrant nutritional environment during the periconceptional, fetal or early postnatal period programs susceptibility to chronic diseases later in life[289]. The results we observed with maternal protein restriction during pregnancy are largely in keeping with those previously reported[90, 290]. As expected, a protein restricted maternal diet resulted in lower offspring birth weight, however, it was not low enough to be considered IUGR which is usually defined as lower than the 10th percentile of normal pups[282]. Maternal PR also affected body composition with PR offspring exhibiting reduced lean mass and higher percent body fat at 4 weeks of age. At 24 weeks of age additional differences were observed, with more negative outcomes noted in male versus female offspring. Male PR offspring had decreased lean mass and naso-anal length and higher AUC for GIP than control offspring. Both male and female offspring from PR dams had decreased liver weight, decreased colonic expression of SGLT1 and increased *C. coccoides* in cecal contents, the latter of which has been previously reported[281]. Although not measured in this study, numerous other detrimental outcomes of gestational protein restriction have been documented including renal disease and hypertension[291]. Given the detrimental effects associated with maternal undernourishment, our objective was to determine if prebiotic fiber intake could mitigate some of the negative metabolic effects of gestational protein restriction.

Offspring of control dams exhibited many of the known benefits of consuming prebiotic fiber. Male offspring had reduced body weight and absolute fat mass similar to that shown in a variety of rodent models[25, 112, 182, 219] and in humans[19]. Female offspring had increased lean mass and colon length and a reduction in plasma levels of LPS, a factor associated with metabolic endotoxemia and the low grade inflammatory tone of obesity[25, 219]. In both sexes, the fiber was associated with a decrease in percent body fat and an increase in cecum weight, PYY AUC and plasma GLP-2. Increases in the anorexigenic peptide PYY has been shown in both rodents[117] and

humans[19] with increased prebiotic fiber intake and may be linked to the reduced energy intake experienced in response to the fiber[117, 118]. Finally, the prebiotic fiber diet also modulated the gut microbiota with characteristic increases in *Bifidobacterium* and *Bacteroides* and decreased Clostridiales[25, 182, 183].

The novel question, however, was whether or not consuming the prebiotic fiber from weaning onwards would mitigate any of the detrimental metabolic effects of gestational protein restriction. In fact the high prebiotic fiber diet did improve some of the negative effects of exposure to the low protein diet *in utero*. Insulin resistance has been shown to be increased in rats exposed to fetal malnutrition[292]. As an indication of reduced insulin resistance, the HOMA-IR scores of male offspring that consumed the fiber diet were improved over the PR offspring that were weaned onto a control diet. This improvement is potentially due to the decreased fasting insulin and likely lower insulin throughout the OGTT seen in the PRF male offspring compared to controls. While HOMA-IR scores have been shown to improve with a moderate intake of the prebiotic fiber inulin in healthy subjects[293], the improvement in insulin resistance following gestational protein restriction is a novel finding. Improvements in glucose tolerance could in part be due to the decreased fat mass seen in PRF offspring or increased production of the short-chain fatty acid (SCFA) propionate. As a fermentation by-product of prebiotics, propionate has been shown to decrease fasting blood glucose and inhibit gluconeogenesis in hepatocytes[294].

Mounting evidence suggests that certain diseases are associated with specific gut microbiota signatures[295, 296]. In many diseases, such as in obesity, levels of bifidobacteria have been shown to be reduced although not unanimously[297, 298]. Confirming previous work we demonstrated that the prebiotic fiber diet increased *Bifidobacterium* in the cecal contents; a result that is of particular importance for the PR offspring given previous reports of decreased *Bifidobacterium* in IUGR rats[281]. This increase in bifidobacteria may also be linked to the observed improvement in HOMA-IR scores based on work showing an association between increased bifidobacteria and normalized fasting insulin in rats fed a prebiotic supplemented high fat diet[25]. The increase in *Bifidobacterium* was greater in male rats consuming the fiber versus females

which may explain why the same improvements in HOMA-IR were not evident in female offspring.

It is well known that maternal malnutrition compromises organ growth in offspring[4]. In regards to the intestinal tract, IUGR offspring have a thinner intestine and smaller surface area for nutrient absorption resulting in reduced growth and compromised health[299]. In our model, offspring from control dams that consumed the fiber had increased small intestine length and females also had increased colon length. In animals from low protein dams, colon length was increased in those consuming fiber. While increased colon length and/or mass is a common feature of prebiotic fiber consumption observed in animals[166], the correction of the typical IUGR-associated short colon phenotype[282] suggests the fiber may have aided in overcoming this developmental deficit.

While the prebiotic fiber diet had beneficial effects on metabolic markers related to obesity and type 2 diabetes (i.e. adiposity and insulin resistance), the finding related to increased LPS in female PR offspring consuming the fiber warrants further examination given the links established between circulating LPS, compromised gut barrier function and metabolic endotoxemia[25, 219]. These findings could provide an interesting perspective regarding the increasing incidence of metabolic disease and type 2 diabetes in developing nations[300]. Particularly in rural areas, protein intakes during pregnancy contribute a minimal amount to total energy intake[301]. Children resulting from these pregnancies often then consume a high fiber diet. Individuals in rural China have been reported to consume as much as 76.6 g/day of fiber[302]. While this may not indicate prebiotic fiber intake, if dietary fiber on the whole has similar effects as seen in the present study, intestinal factors such as LPS may influence whole-body inflammation and therefore development of disease[243].

Although we did not measure intestinal permeability directly, Cani et al.[219] showed that plasma LPS and TJP1 mRNA correlate with direct measures of gut barrier function. Animals from PR dams consuming fiber demonstrated evidence of a ‘leaky gut’ as females had increased levels of plasma LPS and had decreased expression of TJP1 in the colon compared to control offspring consuming fiber. Males also had decreased

colonic TJP1 expression compared to PR offspring that consumed control diet but LPS levels did not differ between the groups in males. Other factors involved in the integrity of the gut are the trefoil peptides, specifically Tff3 and the related MUC2, which are present in the colon and play a role in protecting the epithelium and restoring mucosal barrier integrity after injury[303]. In infants with necrotizing enterocolitis, which is more frequently found in pre-term and small for gestational age infants, Tff3 is found to be down-regulated[303], as it was in our offspring from PR dams. Similarly, MUC2 has been shown to be decreased in PR offspring at weaning[282] which is consistent with our female PR offspring. MUC2 protein treatment has been shown to decrease bacterial translocation, and protein levels are found to be lower in children with Hirschprung's-associated enterocolitis[304]. In this study the prebiotic fiber did not have an effect on the expression of these restorative genes. In other work, Garcia-Rodenas et al.[286] showed improvement in the intestinal barrier in maternally stressed pups when weaned onto a diet that included a 4% dose of the prebiotic fructo-oligosaccharide, however this diet also included galacto-oligosaccharides, probiotics, and long chain poly-unsaturated fatty acids. Taking into account our results, it is possible that the beneficial effect on the intestinal barrier described by Garcia-Rodenas et al.[286] was a result of one of the other supplements, or the synbiotic effect of the ingredients working in concert. Despite the consistency of our findings in regards to markers of intestinal permeability (plasma LPS, gene expression), a direct *in vivo* measure of permeability, such as the FITC dextran or sucrose/lactulose/mannitol protocol, was not performed and the functional significance of these findings remains to be confirmed.

One limitation of the study was the unexpected morbidity and death of some of the fiber-fed rats. While a 21% fiber diet is considered a high fiber diet it is within the range of fiber doses (0-30%) previously tested in rats[305, 306]. We have also utilized this precise diet in previous studies with weanling Wistar rats without problem and in fact observed benefits in terms of reduced body fat and glycemia[113, 117]. Furthermore, we recently showed that a maternal diet with 21% prebiotic fiber was associated with reduced plasma LPS in female offspring at 22 weeks of age (Hallam et al., unpublished results). For unknown reasons the 21% diet was not tolerated as well by the cohort of

animals in this study and therefore we reduced the dose to 10% at 10 weeks of age. No further deaths occurred after this point. While the deaths occurred in offspring from both control and PR dams, the already compromised intestinal structure and function of the PR offspring may have exacerbated their response and could be linked to the higher LPS seen in PRF female offspring. Bacterial translocation was previously shown to increase in artificially reared rat pups with supplementation of galactooligosaccharides and inulin at postnatal day 18, although it was no longer present at day 40[307]. Since prebiotic fiber can increase total bacteria in the gut[166, 182] it is possible that the combination of compromised intestinal structure and function in PR offspring and increased bacterial load explains the higher plasma LPS seen in female PRF offspring. Numerous genes have been identified to play a role in protecting the immature neonatal gut of IUGR animals including B4GALT6, TNFSF13, ICAM2, CPN2, DPP7, LGALS2, PTPRCAP, BCL10 [308] and their examination in our model could help explain the unexpected results.

Based on findings that GLP-2, a gut trophic factor, reduces translocation of LPS[219], we would have expected that the increase in portal GLP-2 we saw in control and PR offspring with fiber intake would reduce LPS translocation. In male and female offspring, GLP-2 levels were increased with fiber in both control and PR offspring although the magnitude of the increase was greater in male PR rats than female PR rats; potentially explaining the sex-specific effect on plasma LPS. If there were differences in GLP-2 at early time points, such as when the animals died between weeks 7-9, it is possible that the adult measures of GLP-2 were not a good indicator for early GLP-2 response in the offspring. It has been shown in pigs who have undergone a small bowel resection that GLP-2 levels rose over the first two weeks post-operatively, stayed approximately the same for another two weeks, and then declined over the following 4 weeks[309].

In conclusion a high prebiotic fiber weaning diet after maternal protein restriction had differential effects on correcting adverse *in utero* programming. Beneficial effects related to metabolic disease included a reduction in fat mass in both sexes and an improvement in HOMA-IR scores in males. Markers of intestinal barrier function,

however, as measured by tight junction protein mRNA and plasma LPS, were impaired in female offspring of PR dams following prebiotic fiber consumption. While the mitigation of some of the detrimental metabolic effects of a protein-restricted maternal diet occurred with prebiotic fiber in the offspring, further work is warranted to understand the functional significance of the changes in markers of intestinal permeability observed in this study.

Chapter Seven: Discussion & Conclusions

7.1 Introduction

Obesity and the associated co-morbidities of type 2 diabetes and cardiovascular disease are major health concerns of the twenty-first century with both personal and economic consequences. Evidence continues to accumulate demonstrating the developmental origins of these diseases. Past research provided evidence for the association between folate deficiency in early pregnancy and neural tube defects, vitamin D deficiency and rickets, smoking during pregnancy and low birth weight and alcohol consumption and fetal alcohol syndrome. This knowledge brought about direct changes to the management of pregnancy, with folate and prenatal vitamins being recommended to prevent neural tube defects and rickets, and a change in public opinion and strong recommendations for abstaining from smoking and alcohol during pregnancy. It is now equally important that evidence regarding maternal dietary intake and the epidemics of chronic metabolic disease be generated in magnitude sufficient enough to inform revised nutritional guidelines for macronutrient intake during pregnancy.

High protein diets are often encouraged both by popular media and medical professionals for the purpose of losing weight, or treating certain conditions[18]. Meanwhile the amount of fiber in the average western diet is much lower than the recommended intake level despite evidence of various benefits such as improving postprandial glycemia and reducing blood cholesterol levels[21, 178, 179, 310]. Prebiotic fiber in particular also has beneficial effects on the gut microbiota which have been linked to overall metabolic health[24]. The long term effects of these diets when consumed during pregnancy have not been fully examined.

Also of interest is the reversal of negative programming that has occurred in the gestational period. Evidence has shown plasticity of newborn animals[36, 49] and beneficial, easy to administer treatments, such as dietary interventions, could be very helpful in improving long-term health of an individual despite an adverse environment encountered *in utero*.

The research presented above was intended to gain a greater understanding of how these diets, when consumed during pregnancy, growth, or in adulthood impact physiological functioning of adult offspring. This was done through the examination of physical characteristics, gut microbiota and milk composition of dams, as well as the physical characteristics, plasma satiety hormone concentrations, gene expression of selected genes involved in glucose and lipid metabolism, and the gut microbiota of the offspring in adulthood.

7.2 Strengths and Limitations

7.2.1 Animal Model

Rodents are a useful model in developmental programming research due to their short gestational period and rapid growth into adulthood. In using an animal model for programming, a more uniform life experience is provided due to the control of the laboratory environment. This controlled environment minimizes the influence of extraneous life events in the programming model; an important consideration given that maternal nutritional status is impacted both by immediate environment and that encountered throughout life to that point[3]. Because the period of time prior to conception is longer than the period of gestation and lactation, the "clean slate" in animal models at the commencement of the experimental period is advantageous[3]. There are some drawbacks in using a rodent model, however, especially in regards to translation to humans. Hypothalamic development in rats continues after birth through the suckling period[311]. Humans are a precocious species, as hypothalamic development and arcuate projections are essentially developed at birth[311]. Humans also have a much larger and higher functioning brain than rodents. This means that organ growth and development *in utero* may differ between species particularly in response to maternal environments that are suboptimal. In fact, humans may be more greatly impacted by adverse maternal environments as evidence points to brain sparing in situations where nutrition is inadequate for full development[4]. Larger, more developed brains may mean that energy is directed towards the brain and away from other organs such as the pancreas[4].

7.2.2 Dietary Interventions

Prebiotics are defined as "nondigestible food ingredients that beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited number of bacterial species already resident in the colon, and thus attempt to improve host health"[27, 312]. The high prebiotic fiber diet in the above studies consisted of equal parts inulin and oligofructose, both of which require bacterial activity to be broken down. We used a very high dose of fiber in these studies at 21.6% by weight. This dose of prebiotic fiber was used, similar to the high protein diet, to represent a high intake of fiber rather than a normal or adequate intake of fiber. Furthermore, it represents a dose more similar to a diet supplement rather than that achieved strictly by consumption of foods high in fiber. While this exact dose would not be possible for human consumption, it is an acceptable level of fiber in a rodent model due to the anatomical differences of the gastrointestinal tract between humans and rats including the larger cecum and relative absorptive surface area in rats[313]. Because of the larger cecum, rats have a greater capacity for fermentation of non-digestible carbohydrates, such as prebiotic fiber, versus humans, and can therefore handle a larger dose of fiber. Work done in our lab has previously shown a dose-dependent response of satiety hormones in rats from prebiotic fiber[183]. Rats in fact are capable of handling a diet containing up to 30% fiber which was shown to result in the greatest increase in GLP-1 secretion and proglucagon gene expression[305]. With translation to humans, an approximate 5% dose of prebiotic fiber has been shown to illicit increased plasma satiety hormone levels and decreased food intake in overweight and obese adults[19]. The similarity in the physiological effect of the 20% fiber diet in rats and 5% fiber diet in humans in regards to satiety hormone response was the chief selection criterion for the dose of fiber utilized in this research.

It is important to note that prebiotic fiber has not been found to be exclusively beneficial. Some studies have shown that in populations with gastrointestinal disease such as Crohn's disease and irritable bowel syndrome (IBS), prebiotics do not necessarily improve microbial profiles and may worsen certain symptoms[314]. In clinical trials with Crohn's disease patients, neither 15 g/day nor 20 g/day increased bifidobacteria or *F. prausnitzii* and in both trials more participants withdrew from the study when assigned to

the prebiotic arm[128, 129]. A clinical trial using 20 g/day of fructo-oligosaccharides (FOS) to treat IBS found that initially participants in the FOS group did not experience improvements in their symptoms while those in the placebo group did, although at the end of the study there were no differences between the groups[130].

Our protein level was selected in accordance with other groups examining high protein diets during pregnancy in a rat model[114]. Perhaps surprisingly, this high dose in rats is comparable to some estimates of protein intake in pregnant humans with intakes as high as 350 grams of protein per day reported in the third trimester[104]. A diet high in protein consumed by women during pregnancy has been associated with lower birth weight, increased ponderal index, and elevated blood pressure and cortisol levels in the offspring[104, 108-110]. Humans are likely to consume protein from various sources and in differing proportions, though these limitless combinations and permutations would be very expensive to examine in an animal model with the many variables this would introduce. Casein was used as the protein source for all of the diets in this study as it is considered a complete protein source for rats. We have previously shown differences in weight gain in male rats when the protein source in the diet was complete milk protein, soy, whey or casein[315, 316]. It is completely possible, therefore, that using an alternative source of protein in our diet may have yielded different results.

The high fat/high sucrose diet was used in chapters 3, 4 and 5 to increase the degree of dietary mismatch in offspring. This high energy density diet is also able to unmask programmed metabolic dysfunction in offspring that may be latent until exposure to the dietary fat and sucrose occurs[36]. Because this diet is not solely a high fat diet but also incorporates elevated sucrose content, it is a closer approximation of the western diet or the ‘cafeteria diet’ used in other studies where rats are fed the same junk food that humans consume. One advantage of using the formulated HFS diet versus a cafeteria diet is the control of micronutrient composition. The effects of deficiency in one or more micronutrients are well documented[317, 318] and by adequately supplementing the HFS diet with vitamin mix the true effects of the high fat and high sucrose diet can be examined.

Our low protein diet of 8% casein used in the maternal diets of Chapter 6 was based on numerous other studies using the low protein programming model. This diet is known to affect intestinal development[90], insulin sensitivity, blood pressure[89], and serum triglycerides and cholesterol[88] when fed to dams during pregnancy. This model was selected in order to induce the well known effects of a less than desirable nutritional environment during pregnancy in order that any 'reversal' effects of the high prebiotic fiber diet in offspring could be tested.

7.2.3 Length of Interventions

In Chapter 3 diets were started one week prior to mating to allow the full effect of the prebiotic fiber diet to occur during pregnancy. Our HFS dietary challenge was started at an age of 100 days in order to bring the animals past the most rapid stage of growth and into early adulthood. At this point the HFS challenge was initiated and lasted 8 weeks in order to induce obesity. The re-matching period in Chapter 5 was 6 weeks to allow time for possible reversal of the negative effects of the HFS dietary challenge. Indeed, Bartness et al.[258] have shown that the effects of long-term high-fat-feeding (up to 97 days) can be reversed in as little as 4 weeks. Protein restriction in Chapter 6 occurred only during pregnancy in order to explore the specific effects of insult during one critical period, as numerous studies have now shown distinct effects of protein restriction during pregnancy alone, lactation alone or during both[319]. The end point of all studies brought the animals to an age range comparable to other studies looking for a long-lasting programming effect, or reversal thereof[36].

7.2.4 Quantitative Real-Time PCR

Levels of mRNA in various tissues were measured using quantitative real-time PCR (RT-qPCR). RT-qPCR gives insight into specific gene expression that helps provide potential mechanisms of action and can direct further analysis. This is a useful and relatively fast method of determining gene expression, however there are limitations of this technique. mRNA can degrade very quickly and can be broken down by

RNAse's[320]. Efforts made to prevent degradation of the mRNA included excising tissues as fast as possible, snap freezing samples in liquid nitrogen, and keeping samples on dry ice prior to homogenization during RNA extraction. Genes of interest were compared to the reference gene GAPDH, which is not impacted by the dietary interventions employed in this research. Groups were compared using the $2^{-\Delta CT}$ method allowing us to normalize data to the reference gene and compare multiple groups. Samples were also taken at the same time of day (within 2 hours) from all animals to minimize influence of circadian rhythm[321]. Another drawback of using RT-qPCR is that a very small section of tissue is used for determination of gene expression. For the liver, care was taken to examine samples from the same lobe from each animal, however there may be differences between lobes and results from the section of tissue examined may not be representative of the entire organ, as has been seen with some genes in the rat liver and may be related to differing blood supply[322-324]. Changes in gene expression also may not be representative of protein levels coded by the associated gene and may not reflect physiological function therefore further examination using Western blots could be useful. Our gene expression data was used in conjunction with measurements of triglyceride content in the liver and plasma satiety hormones. While this method does provide potential insight into nutrient-gene interactions, the limitations of the application need to be acknowledged.

7.3 Overall Summary and Interpretation of Results

7.3.1 Body Composition

The work presented above highlights the long-lasting impact of maternal diet on the offspring. Most notably offspring body composition is greatly influenced by maternal diet during pregnancy, with changes in gut microbiota and gut development, hepatic lipogenesis, and glycemia also evident into adulthood (Figures 7.1, 7.2 and 7.3).

The high protein and high prebiotic fiber diets in many respects had divergent effects on offspring outcomes; the high protein diet increased body weight and adiposity, while the high prebiotic fiber diet decreased these measures. Increased growth of HP1

animals could be due to a larger dietary mismatch between their suckling period and early adulthood. A high protein diet fed to lactating dams has been shown to result in milk with lower lactose content[137]. This decreased carbohydrate content at a critical developmental period would not prepare the offspring for the high carbohydrate content encountered in the HFS diet in adulthood and the mismatch could contribute to the increased mass and adiposity as has been shown previously[22].

In all three animal studies there was a consistent effect of fiber exposure during pregnancy alone, exposure *in utero* and during suckling, or from weaning into adulthood resulting in decreased growth, whether in length, weight or adiposity or all of the above. In Aim 1 (Chapters 3 and 4) we saw an interesting paradigm with the HF diet. Pups had decreased birth weight and exhibited catch-up growth in their first week, however contrary to what is expected with catch up growth[325], there were no negative effects on body weight and metabolic response observed up to 22 weeks of age. In fact these animals had a number of improved physiological markers compared to HP1 and C1 animals. In Chapter 5, male and female animals on the HF diet were smaller than HP1 and C1 animals, however if compared to animals that had not been exposed *in utero* to the HF diet (RF) there were mixed results as to the benefits of each situation. Here we saw decreased length and a decreased response in weight loss due to re-matching to the HF diet as RF animals lost weight each of the 6 weeks while HF1 animals lost weight only over the first few weeks. This may be because of the already decreased body weight of HF1 animals, or perhaps a decreased physical, or physiological, response to the prebiotic fiber. The decreased response from HF1 animals could be of concern if offspring are less responsive to lifestyle interventions that are often recommended to individuals with early warning signs for metabolic disease such as increased blood pressure, high serum triglycerides and/or cholesterol, or increased fasting or postprandial glucose.

Interestingly in Chapter 3 the difference in body weight and composition between female HP1 and HF1 animals was not due to major differences in food intake, even with increased PYY AUC in HF1 animals. This suggests other mechanisms, such as gene expression, and possibly gene translation were involved in glucose and lipid metabolism

and energy storage. Rehfeldt et al.[106] showed an increased preference for oxidative metabolism in offspring suckled by dams consuming HP. While we were unable to examine energy expenditure in our animals, our HP1 animals had increased plasma NEFA levels indicating an increased availability of fatty acids for oxidation and energy. HF1 animals had lower plasma NEFA levels, and also lower hepatic triglyceride levels despite increased mRNA expression of FAS compared to HP1 animals. Up-regulation of FAS expression could be compensatory considering the lower levels of triglyceride and circulating fatty acids, just as it was lower in HP1 animals with greater fatty acid availability.

Once re-matched to maternal diets there were very clear detrimental effects of the HP diet on lipid metabolism as evidenced by hepatic gene expression. SREBP1c is responsible for regulation of ACC and FAS, all of which were up-regulated by the HP diet in Chapter 5, and are seen to be upregulated in obesity[326]. The reversal in the pattern of FAS expression in HP1 animals is interesting especially considering previous research has shown a decrease in FAS mRNA levels with a low carbohydrate diet[201-204]. Our HP1 animals did not exhibit weight loss with the HP diet, suggesting the continued obesity had a greater impact on this expression than the diet.

In Chapter 6, animals consuming the HF diet from weaning also had decreased length compared to animals consuming control. This is somewhat unexpected as feeding prebiotic fiber at a dose of 10% has previously been shown to increase intestinal calcium, magnesium, iron and zinc absorption, even with increased fecal mass[327]. It is possible that the higher 21.6% prebiotic fiber diet increased fecal mass to a threshold above the 10% diet resulting in a decrease in mineral absorption, although our laboratory has also observed decreases in length in pups weaned onto a 10% fiber diet (Reid & Reimer, unpublished results).

Decreased growth due to a high fiber diet in humans could be seen as a positive or negative effect. In a positive light, a decreased rate of growth could be beneficial in infants, and perhaps more mature individuals, that have started to show rapid weight gain or growth. It could be particularly relevant in infancy and childhood given evidence that rapid early growth is associated with later obesity risk[325]. Animals in Chapter 3

showed that fiber exposure resulted in a decreased risk of immediate consequences of early catch-up growth, although with advanced age more detrimental effects may have emerged. A high fiber diet could also be beneficial in individuals at risk for excessive weight gain during pregnancy, gestational diabetes, or at risk for giving birth to macrosomic babies for other reasons. Epidemiological data has shown that babies born from these conditions are at risk of increased weight and fat mass at birth, increased risk of type 2 diabetes[328], and increased incidence of overweight in childhood and early adulthood[82], therefore introduction of a higher fiber diet may prevent or delay some of these associated diseases.

In contrast, decreased growth could be detrimental in some instances. The intergenerational effects of decreased growth due to high fiber exposure are not known. If decreased growth resulted in smaller organisms, IUGR could result with those offspring being at risk for the many diseases associated with that condition. It has also been shown from epidemiological studies that women of shorter stature are at increased risk of developing gestational diabetes[329]. Therefore numerous aspects of an organism's environmental and physiological status should be weighed when considering prebiotic supplementation to avoid possible negative outcomes.

Overall exposure to a high prebiotic fiber diet seems most beneficial in environments where maternal overnutrition is a possibility. Increased fiber intake could have beneficial effects on offspring at risk whether due to gestational diabetes, increased maternal fat intake, maternal obesity or excessive gestational weight gain. Prebiotic fiber can help slow growth and through the parameters discussed in the next two subsections can have a beneficial impact on offspring metabolism.

7.3.2 Glycemic Control

Differences in measures of glycemic control, whether fasting or post-prandially, are frequently seen in various models of developmental programming. The glycemic axis was a major focus of the initial Thrifty Phenotype Hypothesis put forward by Hales & Barker[35]. In Chapters 3, 5 and 6, glucose and insulin were measured at fasting as well as over the course of an OGTT. Lasting effects due to maternal HF or HP diet in Chapter

3 were minimal with only HOMA-IR score being affected in HP1 animals. It is largely believed that a lasting impact on glucose and insulin occurs in developmental programming due to differences in the HPA axis as well as size, number and function of pancreatic islets[65, 66, 279]. It would appear that these structures were not largely impacted by the maternal diets in our work, and in the liver only PGC1α expression was affected with a down-regulation in female HF1, which would be expected to affect fasting levels of glucose[330], though this was not observed in our study at this time point. However at 22 weeks, animals are still relatively young and in some models abnormal glucose control does not occur until a much greater age[86, 87]. It is important to note that HOMA-IR was used to allow for a simpler method of determining insulin resistance through calculations rather than a euglycemic-hyperinsulinemic clamp. HOMA-IR has been reported to be a better indicator of hepatic insulin sensitivity rather than whole body or peripheral insulin sensitivity[331], therefore there may still be differences in insulin sensitivity of peripheral tissues versus the liver in the animals in our studies.

In Chapter 5 (Figure 7.2), there were no differences in fasting glucose based on diet, although there were effects on fasting insulin and HOMA-IR, with both being greater in male C1 than HF1 or HP1. Glucose AUC was significantly lower in HF1 than C1 animals and HF1 and HP1 had lower insulin AUC than C1. This suggests a possible role for the gut microbiota and/or gut development in regulating glucose control. Plasma LPS levels have been linked to insulin resistance[167], and we saw decreases in this measure in both HP1 and HF1 animals in Chapter 4. Hepatic gene expression of G6Pase was increased in HP1 versus C1 and HF1 animals. This suggests a negative effect of the HP diet as over expression of G6Pase can result in glucose intolerance and hyperinsulinemia[332]. While at 28 weeks these animals had improved HOMA-IR scores and insulin AUC, this may not have lasted with advancing age considering the negative patterns of gene expression in the liver.

Comparing re-matched and un-matched offspring from Chapter 5 also showed significant effects on glucose and insulin. Fasting glucose was higher in re-matched animals (HF1 and HP1) versus un-matched animals. In HF-consuming animals, glucose

AUC was also higher in HF1 versus RF animals and insulin AUC was higher in RF females compared to HF1 females. While HF1 animals did not have as good of glycemic measures as RF animals, they were still improved over C1 animals, which again could be linked to gut microbiota, their effects on LPS and subsequently insulin resistance[167].

Many of these outcomes were unexpected. The mismatch theory postulates that the nutritional environment is predicted based on that experienced in utero, and therefore if they do match, maximal health will result[2]; although there does seem to be a caveat that matching requires a certain level of healthfulness in the diet as maternal fat intake followed by pup fat intake at weaning or later in life still has detrimental effects[38, 43, 44]. Both RF and RP animals had better fasting glucose levels than HF1 or HP1 animals. Fasting glucose is a measure often used to determine metabolic health, and is one of the criteria established for diagnosing metabolic syndrome[333]. The improved levels in reference animals suggest a greater impact of the HP and HF diets after the HFS challenge. This could be due to a greater physical and physiologic adaptation required because it is their first encounter with the macronutrient composition. If diets such as these are successful in improving metabolic markers and health because of adaptation in the body, it calls into question the advisability of following any particular diet other than the most balanced possible if it means future disease states would be more difficult to treat, though it is not known how these animals would react to pharmacological interventions. While the HF1 animals appeared to show beneficial, preventative effects in Chapters 3 and 4 it is unknown how long these effects could last if a period of unhealthy lifestyle choices was prolonged.

That being said, animals that consumed HFS for the longest period had improved glucose measures at fasting and over the OGTT, as well as lower fasting insulin levels. This is certainly contrary to expectations as generally a high fat diet is associated with increased lipid accumulation in tissues that then influence insulin sensitivity[334]. A possible explanation is the lower energy intake of H animals versus C1, as well as the lower carbohydrate content of the HFS diet compared to the control (AIN-93M) diet, as diets with a lower carbohydrate content are associated with improved measures of glycemic control[254].

In Aim 4, HOMA-IR scores in males were increased with maternal protein restriction, but decreased to a level not significantly different from controls with consumption of the HF diet. In PRF males, insulin AUC was also lower than PRC males. Males are frequently shown to be more greatly impacted by maternal restriction of any kind during development than females[335]. It is likely, based on published evidence that maternal energy restriction during gestation resulted in developmental defects of the HPA-axis and pancreatic islets[336, 337]. Higher insulin secretion could indicate some level of insulin resistance that could likely worsen with advancing age[337].

7.3.3 Gut Microbiota

Increases in *Bifidobacterium* spp. with prebiotic fiber exposure was a consistent finding where gut microbiota was measured. This species has repeatedly been shown to increase with consumption of prebiotic fiber[27], and is often associated with improved measures of metabolic health such as decreased endotoxemia, improved glucose tolerance and glucose-induced insulin secretion and decreased measures of proinflammatory cytokines in plasma and adipose tissue[25].

In Chapter 4, there was a significant increase in the proportion of *Bifidobacterium* spp. in HF1 males compared to HP1 and C1 males, and in HF1 females versus HP1 females. Importantly this was observed at the age of 22 weeks when the HF diet exposure occurred only during gestation and lactation, along with any diet ‘sampling’ by the pups that may have occurred prior to 3 weeks of age before separation from the dam. The increased proportion of *Bifidobacterium* spp. was also evident after a high fat/sucrose challenge. A high fat diet is known to have detrimental effects on the gut microbiota with decreases in total bacterial numbers, the *E. rectale-C. coccoides* group and *Bifidobacterium* spp., while increasing proportions of Bacteroidales and Clostridiales[25, 169].

While gut microbiota was not measured in Chapter 5, evidence such as plasma levels of GLP-1[338], differences in HOMA-IR scores and body weight point to possible influences of the gut microbiota. As these animals were also from the dams used in Chapters 3 and 4, the maternal influence over the development of the gut microbiota that

was still evident at 22 weeks was likely to still be present at 28 weeks of age. A high prebiotic fiber diet is known to increase *Bifidobacterium* spp.[27] and this increase would be anticipated in both HF1 and RF animals. A high protein diet has been shown to decrease numbers of *Bifidobacterium* spp., which would be expected to be a factor in both HP1 and RP animals[29, 30]. There is increasing evidence that animals are not born with a sterile gastrointestinal tract and that colonization of the fetal gastrointestinal tract does occur based on the microbial community of the mother[153]. Bacterial transfer also appears to continue after birth via milk consumption[34]. Given the results of the milk oligosaccharide analysis, there are certain factors that reference animals would not have been exposed to, giving reason to believe that especially between RF and HF1 animals there would have been differences in the make up of the gut microbiota, particularly in regards to *Bifidobacterium* spp., as it is likely HF1 animals would have started life with a greater number of *Bifidobacterium*, and that this community would have been nourished by the oligosaccharides from maternal milk[246]. There may also be differences in the make up of the bifidobacteria in the gut of HF1 versus RF animals because of the different oligosaccharide exposures neo-natally resulting in different abilities to thrive off of inulin and oligofructose[246]. As similar increases in two milk oligosaccharides occurred with both the maternal HP and HF diet, different responses in RP and HP1 animals could also be present. Milk oligosaccharides are often associated with increases in *Bifidobacterium* spp.[238, 239] however without the prebiotic effect of the fiber, HP1 and HF1 animals did not have similar microbial profiles at 22 weeks of age after a high fat high sucrose dietary challenge. With exposure to increased levels of certain milk oligosaccharides different gut microbiota responses to the HP diet may have occurred between HP1 and RP animals.

In Chapter 6 (Figure 7.3), there were significant increases in *Bifidobacterium* spp. with consumption of the HF diet, as expected. With protein restriction *in utero* there were even greater increases in *Bifidobacterium* spp. Unfortunately, the generally positive associations of increased Bifidobacteria, such as decreased measures of intestinal permeability[25], were not evident in PRF offspring, and were in fact increased in females. Recently other members of the gut microbiota have been found to play an

important role in gut barrier function. *Akkermansia muciniphila* has been found to be important for reducing metabolic endotoxemia, and decreases in obesity and type 2 diabetes[168]. While in obese and type 2 diabetic mice prebiotics can restore *A. muciniphila* levels, it is not known how maternal protein restriction and potentially compromised gut function would interact with prebiotics and *A. muciniphila,* and previous studies have found that prebiotics are unable to increase *Bifidobacteria* in patients with Crohn's disease[128, 129].

It is also possible that the loss of some of the beneficial effects may have been due to different milk exposure. In Chapter 4, there were increases in milk oligosaccharides that are associated with development of the intestinal microbiota and preventing adherence of pathogens to the epithelial surface of the intestine[241, 339]. Research has shown that there is a marked impact on offspring of protein restricted mothers if that restriction occurs during lactation[88]. In our study, protein restriction only occurred during gestation and we did not measure any components of the milk between the two groups. Protein restriction during lactation has been shown to decrease milk protein concentration compared to rat dams returned to a normal diet at parturition[340] (Pine et al., 1994) so effects on offspring of PR dams in our study due to milk composition are not very likely. It is possible though that a difference may have existed in the oligosaccharide composition at some point in the lactation period.

Bifidobacteria has been repeatedly associated with beneficial measures of health[25, 167, 243]. In Chapters 3 and 4, as well as Chapter 6, there were benefits observed with increased *Bifidobacterium* spp. in response to exposure to the HF diet at some point from conception to post-weaning, especially in regards to measures of glycemic control. However given some other questionable outcomes seen in Chapter 6 with the HF diet, further investigation - into the dose and timing of the fiber consumption, as well as possible contraindications for use - is needed to ensure maximal beneficial effects while avoiding intestinal changes that could potentially have metabolic harm with increasing age.

Figure 7.1. Summary of major findings in adult offspring of dams consuming a high prebiotic or high protein diet

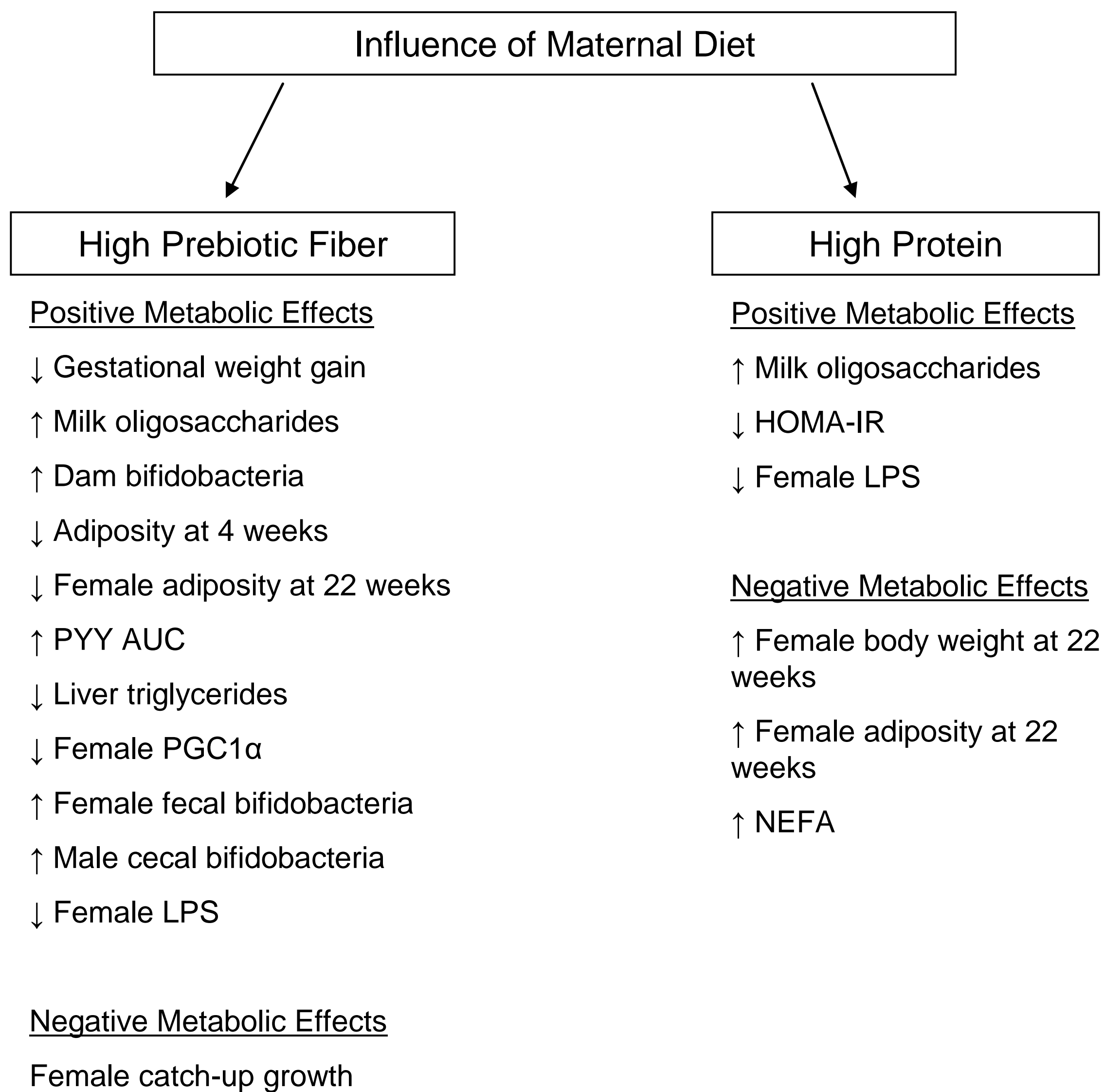

Figure 7.1. Overall summary of the major findings in adult offspring after a high fat/sucrose dietary challenge due to a maternal diet high in prebiotic fiber or protein.

Figure 7.2. Summary of re-matching adult animals to maternal diet

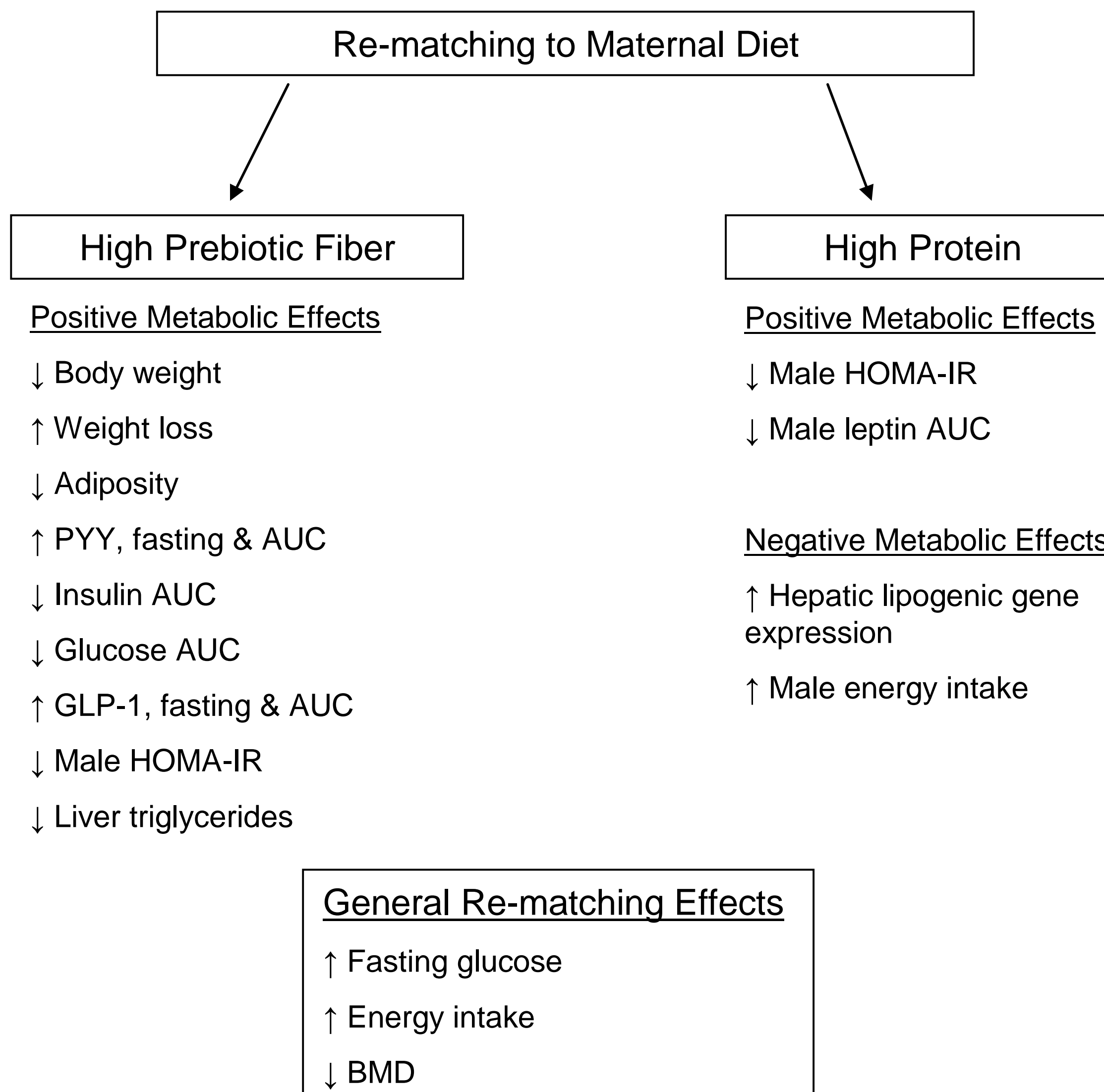

Figure 7.2. Overall summary of the major findings due to re-matching animals to the diet they were exposed to maternally.

Figure 7.3. Summary of effects of a weaning diet high in prebiotic fiber with the effects of maternal diet

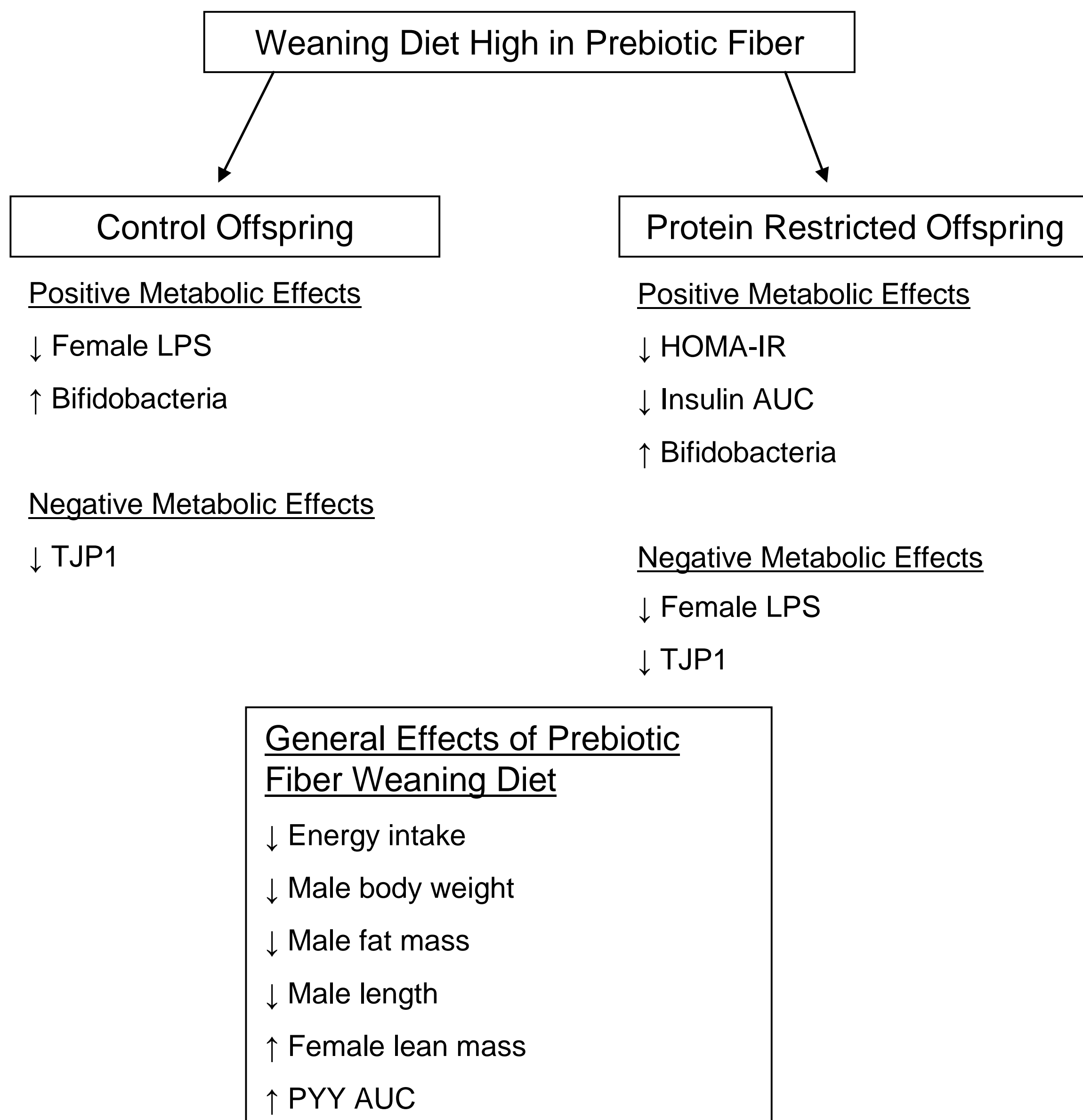

Figure 7.3. Summary of the major findings for a weaning diet high in prebiotic fiber when given to pups from dams consuming control or protein restricted diet during pregnancy.

7.4 Future Directions

The above studies highlight both positive and negative possibilities of dietary manipulation during pregnancy and in early post-natal life. The demonstration of possible prevention of detrimental effects from an adverse diet in adulthood as well as the reversal of negative programming from the gestation period provides promise for improvements in early developmental nutrition. However there are many other aspects that can be investigated and a number of mechanisms that need to be better understood.

In regards to Chapters 3 and 4 more work is needed examining the early impact of maternal diets high in protein, and especially prebiotic fiber. While likely more relevant in application to humans, because dams were fed their experimental diets during both pregnancy and lactation, it is difficult to say which period had the most influence on future health of the offspring, or if the combined period of exposure was important to achieve the observed effects, particularly in regard to female anthropometrics. Evidence from examining differing protein levels in the offspring have shown that a larger impact occurs with exposure during the suckling period, though this could be due to the continued development of arcuate projections in the hypothalamus during suckling in rodents[88, 137, 311]. As the intestine plays such a large role in overall health due to its importance in nutrient absorption as well as playing host to the large number of bacteria, this organ needs to be well understood during this important period of rapid growth and change. Our results from Chapter 6 showed potential lasting effects of the maternal diet on the intestine and an interaction of prebiotic fiber with various measures related to the intestine. For example, prebiotic fiber was shown to increase GLP-2, which was not measured in Chapters 3, 4 and 5 though repeated measurements during early life as well as in adulthood could aid in understanding the physiological changes that occur with a maternal diet high in prebiotic fiber. It would also be interesting to examine changes in the gut in response to the HFS diet, looking at gut permeability and morphology prior to HFS consumption giving a greater idea of any protective effects of the maternal prebiotic fiber diet.

Longitudinally, more investigation is also needed in regards to all animal studies presented in this thesis. It has previously been reported that animals consuming prebiotics throughout their lives live longer[198], and in some programming models effects have been observed up to twelve generations later[1]. As our studies went to a maximum of 28 weeks we do not have any information as to the longitudinal effects into senescence of the maternal diets. Also of importance is the reproductive fitness of offspring exposed to our maternal diets. Adaptations that occur *in utero* can affect growth and development of numerous organs as well as body size in general which could impact ability to conceive as well as the amount of investment the next generation, particularly females, are able to make in their own offspring[3]. In Chapters 5 and 6 there were experimental dietary periods during the life of the offspring as well which could influence their reproductive fitness and impact their offspring's lives.

Further investigation into the effects of our experimental diets on maternal milk production is also warranted given the differences in oligosaccharide composition associated with our diets. Examination of mammary gland tissue could provide further information about the impact of dietary components on glycosyltransferase enzyme activity[341]. The effect of these dietary components on human milk composition would warrant examination.

Our maternal high protein diet, while increasing oligosaccharide content of maternal milk and improving HOMA-IR scores in adult offspring, was generally detrimental with increased food intake resulting in increased body weight and adiposity. In other experimental designs, both human and animal, high protein diets have been shown to aid in weight loss and normalization of certain blood markers[342], and have also been found to be beneficial when fed to dams nursing cross-fostered pups exposed to food restriction in utero[343]. It would be interesting to examine the effects of a maternal diet high in both fiber and protein to determine if they may have a synergistic relationship or to see if the prebiotic fiber may be able to prevent some negative effects of the maternal HP diet. Over 3 weeks a diet high in both protein & prebiotic fiber was previously shown to increase GLP-1 secretion in genetically obese rats[188] which over time could affect body weight. A weaning diet high in prebiotics may also be interesting

after maternal HP exposure to examine the effects on gastrointestinal function and weight gain, especially given the effects observed in Chapter 6 after maternal protein restriction.

More evidence has been presented recently showing a lasting impact of paternal lifestyle on their offspring[344]. Negative effects, including increased adiposity, impaired glucose tolerance and insulin secretion[345], could potentially be corrected by paternal prebiotic fiber consumption. There is evidence that protection could be transferred via paternal influence as breeding with male rats in the fasted state can decrease plasma glucose in offspring[346].

As with most animal research the ultimate goal of the above research would be to translate the findings to human clinical studies. There are many ethical concerns, however, when contemplating interventions in pregnant women and/or neonates, especially in light of evidence of the lasting effect the environment has on both male and female offspring[51]. Randomized control trials have often focused on women with higher-risk pregnancies. A large number of randomized control trials have been conducted in women with gestational diabetes, however all have used varying diet compositions and most have a small number of participants and have not been repeated[347]. Others have examined malnutrition and increasing energy and protein intakes, which are not largely relevant in the current western world[348]. There are so many factors to consider when examining maternal nutrition and child health that more research is required to optimize health[51]. While the results of the above studies may not warrant randomized control trials in pregnant women it does provide data for potential guidelines for evaluating the diet of pregnant or nursing women, as well as recommendations for feeding infants during the first year of life, especially taking specialized circumstances into consideration as data has shown better pregnancy outcomes with dietary counselling during pregnancy[349, 350].

7.5 Conclusions and Significance

The above work showed an influence of prebiotic fiber on the growth and health of adult offspring. Whether consumed during pregnancy and lactation or post-weaning

there were numerous beneficial effects on body weight, adiposity, insulin sensitivity and gut microbiota. In contrast, manipulation of the protein content of the diet during pregnancy and lactation has adverse effects on offspring health with body mass and composition, insulin sensitivity and gut microbiota being affected based on the restriction or excess of protein. Encouraging consumption of a maternal diet with adequate or perhaps even high fiber intake and adequate protein intake could be beneficial in controlling weight gain during pregnancy. Further investigation into the ideal timing and dosage is needed to ensure maximal benefits without adverse side effects.

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APPENDIX A: IMPACT OF MATERNAL DIET ON COLON MORPHOLOGY

A.1. Introduction

The gastrointestinal tract is a series of organs displaying a vast degree of plasticity. Structures and functions such as motility, biofilms and wall thickness can adapt based on feeding, disease status or nutritional composition[351-353]. Specifically, changes in wall thickness, including the muscle, mucosal and submucosal layers, affect the stiffness of the intestine and can be measured biomechanically. Stiffness is generally measured bi-axially, in the circumferential and longitudinal directions. The intestine is stiffer longitudinally than circumferentially, and they do not necessarily change together[352].

Development of the gastrointestinal tract begins early in gestation, in humans in the second week, with the colon differentiating at 6-7 weeks[354]. During fetal development the gastrointestinal tract is formed from the endoderm and is surrounded by mesenchyme. It starts as a primitive tube developed in a cranial-caudal direction that transitions to a columnar epithelium wherein enterocytes develop along with the brush border and basolateral membranes[355]. Development and later function can be influenced by the *in utero* environment as infants with gastrointestinal atresias exhibit growth restriction. Varying carbohydrate content in maternal diet affects glucose concentration of the amniotic fluid and similarly changes in maternal diet can influence fatty acid concentration of the amniotic fluid[356-358]. The effects of maternal prebiotic fiber intake on intestinal stiffness have not been examined, nor has a high protein diet during pregnancy and lactation. Based on previous gross anatomical observations of offspring intestine in our lab, we hypothesized that a maternal diet high in prebiotic fiber would result in a thinner, more fragile gut. Based on previously published studies, we also predicted that a maternal high protein diet would not show significant differences from control[353].

A.2. Methods

A.2.1. Animals and Diets

Thirty-seven virgin Wistar dams were obtained from Charles River (Montreal, QC, Canada) and housed in a temperature and humidity controlled facility with a 12-h light/dark cycle. After 1 week of acclimatization, animals were given one of three nutritionally complete experimental diets: high prebiotic fiber (HF) (21.6% wt/wt, 1:1 ratio of oligofructose and inulin), high protein (HP) (40% wt/wt), or control (C) (based on AIN-93G). All maternal diets were mixed in house using ingredients purchased from Dyets, Inc. (Bethlehem, PA, USA); the detailed composition has been previously published[112]. Dams consumed the diets for one week prior to being bred with male Wistar rats in wire-bottomed cages. Following the identification of a copulation plug, dams were housed individually and continued to consume their assigned experimental diet (C, HF, or HP) until the pups were weaned. Dams were weighed weekly, and food intake was measured throughout week 2 of pregnancy. Pups were weighed on the day after birth, and litters then culled to 10 pups with equal numbers of males and females where possible. Offspring were weighed weekly for the remainder of the study. At weaning (3 weeks), 1 male was randomly selected from each litter to continue in the study until 22 weeks of age. Pups were weaned onto AIN-93G control diet[187]. Offspring were then switched to AIN-93M at 10 weeks of age. At 14.5 weeks of age, offspring were fed a high fat, high sucrose (HFS) diet[182] for 8 weeks. At 22 weeks rats were fasted overnight and killed via over-anaesthetisation and aortic cut. The University of Calgary Animal Care Committee approved the experimental protocol and all procedures were conducted in accordance with the Guide for the Care and Use of Laboratory Animals.

A.2.2. Biomechanics

Two centimetre sections were taken from the mid-colon and rinsed in proteinase inhibitor cocktail and snap frozen in liquid nitrogen. Samples were stored at -80ºC until analysis. Samples were then thawed and carefully cut open longitudinally opposite the mesentery. The flat piece of tissue was then carefully clamped into the ElectroForce

Planar Biaxial 2 Motor TestBench Test Instrument (Bose Corporation, Eden Prairie, Minnesota). The sample was kept moist with PBS. A pull to failure program was used pulling at a rate of 0.33 mm/second.

A.2.3. Histology

A 0.5 cm section from mid-colon was fixed in non-buffered formalin. Samples were then embedded in paraffin and non-serial histological 5.0 μm sections were stained with hematoxylin and eosin. Samples were then examined for thickness of the muscularis externa, submucosal and mucosal layers using a light microscope (Zeiss Axiostar Plus, Carl Zeiss Microscopy GmbH 2011, Munich, Germany) and Zen 2011 (blue edition) software (Carl Zeiss Microscopy GmbH 2011, Munich, Germany). Measurements were taken from 4 to 10 slides per animal and then averaged.

A.2.4. Statistics

All data are presented as mean ± SEM. Data collected from the dams was analyzed with one-way ANOVA with Tukey's post hoc analysis. Statistical analysis was performed using SPSS v 20 software (IBM, Chicago, IL).

A.3. Results

A.3.1. Biomechanics

There were no significant differences among diet groups in the force needed to cause failure in either the longitudinal or circumferential directions of the colon (Table A.1).

A.3.2. Histology

There were no differences in thickness of the muscularis externa layer or the mucosal layer of the colon between diet groups. Submucosal layer thickness was significantly greater in HP1 than HF1 animals (P=0.012) (Table A.1).

Table A.1. Force data and histological measurements of male colon.

	Control	High Protein	High Fiber	*P*
Longitudinal Stiffness	1.52±0.29	2.14±0.29	1.71±0.37	0.416
Circumferential Stiffness	0.977±0.21	1.14±0.10	1.31±0.19	0.416
Muscularis Externa	178.45±35.2	205.85±7.59	220.53±17.05	0.407
Submucosa	49.91±2.16^{ab}	60.19±2.05^{b}	40.52±4.49^{a}	0.012
Mucosa	216.59±17.92	240.18±29.70	254.50±21.49	0.451

A.4. Discussion

Measures of intestinal stiffness indicated no differences between diet groups, while intestinal layer thickness was only different in the submucosal layer between HP1 and HF1 animals. As the submucosa is the thinnest of the three layers of the colon it is not surprising that this did not result in changes in stiffness. Submucosa thickness has previously been shown to decrease in mink fed a low protein diet for 6 weeks, while a high protein diet did not affect layer thickness[353]. This low protein diet also resulted in a decrease in circumferential stiffness, while the high protein diet did not differ from the control[353]. Increases in submucosal thickness have been observed with Crohn's disease[359] and small bowel infarction[360] which suggest that our HP1 animals may have had some level of intestinal impairment, though it is difficult to say how significant this effect was, whether it was in a steady-state, improving, or progressing to greater impairment. Long-term effects of a maternal diet have not previously been examined, though given the small differences with direct feeding of various levels of protein[353], and minimal effects on histometry with a maternal high protein diet[90], a lack of change

at the age of 22 weeks is perhaps not surprising. That maternal prebiotic fiber may not significantly change layer thickness is not completely unexpected, as carbohydrate content of the diet did not differ from controls, and in a study with differing carbohydrate levels, despite differences in amniotic fluid glucose, maternal and fetal plasma glucose were not different[356]. Prebiotics have been reported to stimulate epithelial cell proliferation when consumed[361], though continued stimulation is not likely 19 weeks after last exposure to prebiotics as other effects of prebiotic consumption have been shown to last a maximum of 5 weeks[27, 160, 161].

In conclusion, maternal diets high in protein or prebiotic fiber did not have a meaningfully large or lasting impact on the morphology of the colon of offspring. Further measures of intestinal histometry, such as villi height and crypt depth, would provide additional information about the lasting maternal influence on nutrient absorption.