2013-09-13

Effects of a Maternal Pre-pregnancy Dietary and Pharmacological Intervention on Maternal Fecundity and Offspring Health in Sprague-Dawley Rats

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Effects of a Maternal Pre-pregnancy Dietary and Pharmacological Intervention on Maternal Fecundity and Offspring Health in Sprague-Dawley Rats

by

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A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE

DEGREE OF MASTER OF SCIENCE

FACULTY OF KINESIOLOGY

CALGARY, ALBERTA

SEPTEMBER, 2013

Abstract

The objective of this study was to determine if a combined oligofructose and sitagliptin pre-pregnancy intervention could mitigate the decreased fecundity, poor pregnancy outcomes and negative offspring health outcomes associated with maternal obesity. Obese female Sprague-Dawley rats were randomized to 1 of 6 groups: 1) AIN-93 (Control-Treated Obese); 2) OFS (Fibre-Treated Obese); 3) Sitagliptin (Drug-Treated Obese); 4) OFS+Sitagliptin (Combination-Treated Obese); 5) Caloric restriction (Weight-Matched to group 4), 6) High fat & sucrose (Obese Untreated). A lean reference group was also included. Pups consumed a HFS diet for 6 weeks in adulthood. Reduced blood glucose was seen in sitagliptin offspring and increased short-chain fatty acid receptor expression and Bifidobacteria seen in offspring of the combination treated dams. Despite these changes, the pre-pregnancy treatments investigated in this study did not lead to meaningful improvements in maternal fecundity, pregnancy outcomes or offspring health.

Acknowledgements

First and foremost, I would like to acknowledge my supervisor, Dr. Raylene Reimer for giving me space to grow and stretch myself during my thesis project and for her tireless efforts in helping me succeed.

I would also like to thank my supervisory committee Dr. Jane Shearer and Dr. Carol Huang for their support, guidance and honesty throughout this process.

The completion of this project would not have been possible without the assistance of all of my lab colleagues especially Kristine Lee, Megan Hallam, Amanda Eslinger and Kendall Schafer.

Finally, I would like thank my family for loving me and being proud of me no matter what.

To my Husband & Family

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List of Abbreviations

Abbreviation	Definition
A	Adverse
AIN-93G	AIN-93 Growth
AIN-93M	AIN-93 Maintenance
AMPK	Adenosine monophosphate-activated protein kinase
ANOVA	Analysis of Variance
ART	Assisted reproductive technology
BMC	Bone mineral content
BMD	Bone mineral density
BMI	Body mass index
C	Control
CR	Caloric restriction
CRP	C-reactive protein
DIO	Diet-induced obese
DIO-P	Diet-induced obese prone
DIO-R	Diet-induced obese resistant
DNA	Deoxyribonucleic acid
DPP-IV	Dipeptidyl peptidase-4
DXA	Dual energy x-ray absorptiometry
F	Females
FDA	Food and drug administration
FSH	Follicle stimulating hormone
GIP	Glucose-dependent insulinotropic polypeptide
GLP-1	Glucagon-like peptide 1
GLUT2	Glucose transporter 2
GnRH	Gonadotropin releasing hormone
GPCR	G-protein coupled receptors
GPR	G-protein coupled receptors
GWL	Gestational weight loss
HDL	High-density lipoprotein
HFD	High fat diet
HFS	High fat & sucrose
HFS-CON	High fat & sucrose control
HOMA-IR	Homeostatic model assessment of insulin resistance
IGF	Insulin-like growth factor
IUGR	Intrauterine growth restricted
IVF	*In vitro* fertilization
Lean-CON	Lean control
LGA	Large for gestational age
LH	Lutenizing hormone
LPS	Lipopolysaccharide
M	Males

mRNA	Messenger ribonucleic acid
NEFA	Non-esterified fatty acids
NL	Normal litter
OGTT	Oral glucose tolerance test
OFS	Oligofructose
OFS+S	Oligofructose and sitagliptin
PCOS	Polycystic ovarian syndrome
PYY	Peptide tyrosine tyrosine
qPCR	Quantitative polymerase chain reaction
RCT	Randomized control trial
rRNA	Ribosomal ribonucleic acid
S	Sitagliptin
SCFA	Short chain fatty acids
SEM	Standard error of the mean
SGA	Small for gestational age
SHBG	Sex hormone binding globulin
SL	Small litter
T2D	Type 2 diabetes
TTC	Time to conception
Txt	Treatment

Epigraph

“Truth, like gold, is to be obtained not by its growth, but by washing away from it all that is not gold.”

~ Leo Tolstoy

Chapter One: **Introduction**

1.1 Background

Around the world, obesity rates are on the rise. According to the World Health Organization, 35% of adults over the age of 20 are overweight and 12% are obese across the globe (1). Globally the prevalence of obesity has doubled since the year 1980 (1). Obesity can be defined as a body mass index (BMI) greater than or equal to 30 kg/m^2, while overweight is considered a BMI between 25-29.9 kg/m^2. Canadians are no exception. Based on self-reported BMI, approximately 50.8% of adult Canadians were overweight and obese in the year 2007. In the year 2011, 52.1% of Canadian adults were considered overweight or obese (2). While the increase of 1.3% may seem minor, it represents an additional 1.08 million people at an unhealthy body weight. Furthermore, roughly, 22% of Canadian youth between the ages of 12-17 are currently overweight and obese (2). In 2009, 19.7% of youth were overweight and obese, a difference of nearly 16,000 youth (2). In total, in the year 2011, more than 13.6 million Canadians were considered overweight or obese. The increasing prevalence of overweight and obesity in the population is associated with numerous negative consequences.

Obesity is a risk factor for the development of an extensive list of diseases and health complications (3-7). The diseases related to obesity span the entire body, ranging from mental illness to diabetes to osteoarthritis (3). Obesity is associated with an increased risk of cardiovascular disease, coronary artery disease, stroke and hypertension (3, 4). There are 14 types of cancer that are linked to obesity, some of which are considered fatal (3). More recently, obesity has been linked with a decrease in fecundity, which is the physiological ability to reproduce (5-8). Obesity-related infertility involves an increased time to conception and decreased implantation in both natural and assisted reproduction (7, 9). Increasing BMI has also been associated with negative obstetrical outcomes (10). Unfortunately, research has also shown that the negative effects of maternal obesity can be passed on to their children.

In the year 2010, approximately 42 million children under the age of 5 were overweight across the world. The transmission of overweight and obesity from mother to

child, or the transgenerational cycle of obesity, may be the result of a phenomenon called "programming". Programming refers to the beneficial or detrimental repercussions that the early life environment can have on adult physiology (11). Programming is also called the developmental origins of health and disease and is defined as an "event during a critical period early in life that results in a persistent outcome for the adult organism" (12). The seminal observation in the programming field was made by Barker et al. (13) when they discovered that poor nutrition during early development was associated with heart disease in adulthood. Programming can only occur at certain stages of development when an organism is in a period of plasticity or in other words is able to change its phenotype based on the environment (14). The current obesity epidemic is thought in part to be a result of a 'mismatch' between *in utero* programming and the current environment of overnutrition (14).

Explaining the programming of obesity is complex because obesity is associated with a vast array of factors including social economic status, environment, gut microbiota and genetics (15-17). For this reason a large variety of obesity interventions have been tested in rodent models and humans including environment, lifestyle, surgery and pharmacology (18). Currently, there is a growing push for weight loss during pregnancy in obese women, but this is highly controversial and there is no clear evidence indicating that it is safe (19). For this reason the intervention utilized in this work was implemented prior to conception. The present thesis work investigates the effects of four pre-pregnancy maternal obesity treatments on offspring health including oligofructose (OFS), sitagliptin, a combination of both OFS and sitagliptin, and caloric restriction.

Prebiotic fibre is an ideal candidate for an obesity intervention because it alters the physiological drive to eat. The most notable prebiotic fibres, oligofructose and inulin, have both been shown to have beneficial health effects when used to treat obesity (20, 21). Consumption of OFS and/or inulin triggers an increase in endogenous GLP-1 secretion in the gut (22). GLP-1 is associated with increased satiety and improved glucose control through the incretin effect (23). Prebiotic intake in both rodent and human models has also been shown to decrease weight gain in overweight and obese subjects and can promote weight loss and loss of fat mass (20, 24). Given the effectiveness of oligofructose in reducing fat mass in

previous rodent and human studies, and its ability to beneficially alter gut microbiota profiles, it was utilized in this thesis work (20, 25). Prebiotics have also been linked to improvements in gut microbiota.

Gut microbiota are altered in obesity wherein the bacteria contribute to increased inflammation and caloric harvest from food (26, 27). Germ free mice that are colonized with microbiota from the gut of obese mice rapidly gain weight and adipose tissue (26). Bacteroidetes, Firmicutes and Proteobacteria are the three main phyla found in the gut with Bacteroidetes and Firmicutes dominating (16). The majority of studies, but not all, have found that the relative abundance of each can be used to distinguish between an obese and normal weight subject (16). Many, but not all, studies suggest that obesity is characterized by an increase in Firmicutes and Proteobacteria and a relative decrease in Bacteroidetes (16, 28). By selectively increasing the abundance of Bifidobacteria in the gut, prebiotics have the potential to shift the gut microbial profile to a lean phenotype.

Pharmacotherapies for obesity have been a target for intense investigation, however, relatively few have proven effective or safe for long-term use (18). The drug intervention used in the present study is a DPP-4 inhibitor, called sitagliptin. Sitagliptin is currently in use in both the United States and Canada for treatment of type 2 diabetes (T2D). Similar to prebiotic fibre, DPP-4 inhibitors work through the incretin system (29). By blocking the enzyme that inactivates GLP-1 *in vivo* DPP-4 inhibitors increase the amount of time GLP-1 remains active in the body (30). Sitagliptin can be prescribed alone or in combination with other known or potential T2D treatments like metformin (29). To date there are relatively few studies that have investigated the combination of a T2D drug and dietary fibre, but with impressive outcomes (e.g. OFS plus metformin and PGX® plus sitagliptin) (21, 31). The combination of OFS plus sitagliptin remains to be examined. The increased production of GLP-1 from fibre and the increased time span for GLP-1 activity provided by sitagliptin may work synergistically to improve glycemia and body weight.

Caloric restriction is a more traditional approach to weight loss. “Dieting” and "cutting calories" is particularly popular in western cultures and people are inundated with advertisements promoting the latest and greatest diet solutions. Caloric restriction is

associated with significant weight loss, but it is also associated with weight regain (32). Caloric restriction during pregnancy and lactation may also result in programming effects in the offspring (33). The present thesis work specifically includes a weight-matched calorically restricted maternal treatment group to isolate which if any programming effects are a result of weight loss alone and which are from exposure to the combined actions of OFS and sitagliptin. Therefore, the weight loss in this group was matched to the weight loss in the combined OFS and sitagliptin treatment group.

1.2 Significance

Obesity rates and infertility rates are on the rise making this project both timely and relevant. The information gained from this study is novel on two fronts. First, it will determine if obesity treatment prior to pregnancy can significantly improve the metabolic outcomes of offspring. Additionally, it will determine if a maternal intervention pre-pregnancy alters maternal fecundity. In a nation where obesity and its associated costs are on the rise, discovery of inexpensive, non-invasive and safe obesity treatments is imperative. Eventual translation of the findings of this study to human clinical studies may enhance the fecundity, metabolic health and health related quality of life for women trying to conceive and their subsequent children.

1.3 Specific Aims

1.3.1 Aim 1 - Maternal

The primary objective for the dams was to determine the effects of a combined therapy of OFS+Sitagliptin prior to pregnancy on the maternal fecundity and pregnancy outcome. It was hypothesized that the OFS+Sitagliptin treatment prior to pregnancy would improve maternal fecundity and pregnancy outcomes in obese female Sprague-Dawley rats.

1.3.2 Aim 2 – Offspring

The primary objective for the offspring was to determine the effects of the OFS+Sitagliptin maternal treatment on offspring metabolic health. Specifically it was hypothesized that offspring born to the OFS+Sitagliptin dams would have improved body composition, glucose control, satiety hormone levels, gene expression and gut microbiota.

1.3.3 Aim 3 – Offspring

The secondary objective for the offspring was to determine the effects a caloric restriction maternal treatment prior to pregnancy on offspring metabolic health. Specifically, it was hypothesized that offspring born to the CR dams would have improved body composition, glucose control, satiety hormone levels, gene expression and gut microbiota.

1.4 Outline

This thesis consists of six chapters describing the experiment outlined in Figure 1.1. Chapter 1 is a brief introduction that highlights the relevant literature, the significance of the work and the specific aims. Chapter 2 is a literature review addressing the maternal and fetal consequences of maternal obesity. A detailed review of the current and potential strategies to offset the negative outcomes of maternal obesity will also be addressed in Chapter 2. The effects of a pre-pregnancy dietary and pharmacological intervention on maternal fecundity will be described in Chapter 3. Both Chapter 4 and Chapter 5 will discuss the offspring outcomes of a pre-pregnancy dietary and pharmacological intervention. Specifically, Chapter 4 will show the results for the four main treatment groups which are: 1) Control; 2) Oligofructose (OFS); 3) Sitagliptin; and 4) Oligofructose + Sitagliptin (OFS+Sitagliptin). The effects of caloric restriction prior to pregnancy on offspring will be the focus of Chapter 5. Chapter 6 will include the general discussion and conclusions of the thesis. References and any additional information in the form of Appendices are provided at the end of the document.

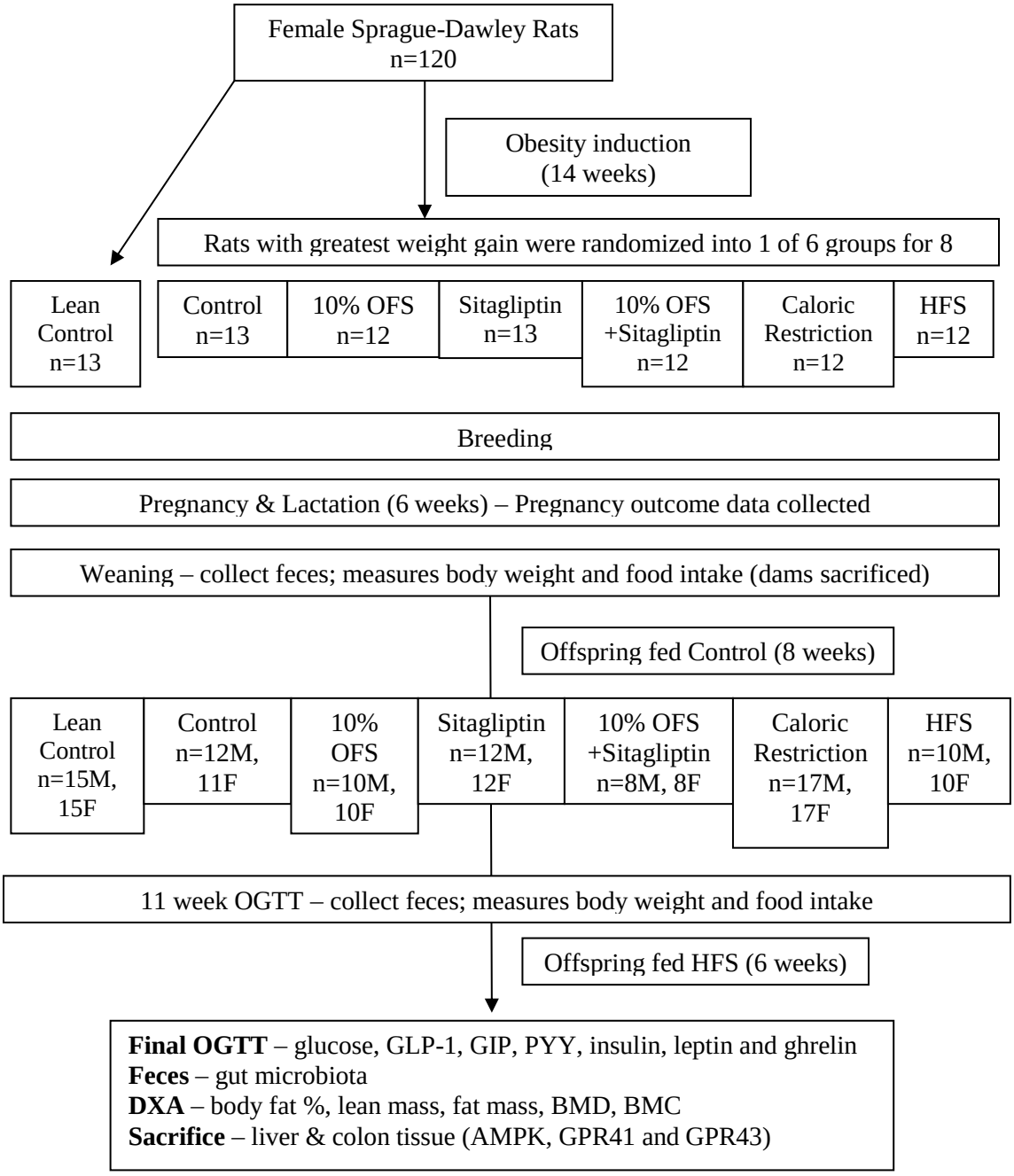

Figure 1.1 Complete experimental design diagram that incorporates all of the maternal treatment groups discussed in Chapters 3-5. The maternal intervention until breeding was completed by former MSc student Amanda Eslinger. This thesis work focused on the maternal fecundity and offspring outcomes.

Chapter Two: **Maternal Obesity: A Cascade of Consequences**

2.1 Maternal Obesity, Pregnancy Outcomes & Fecundity

In 2002, 7.3 million women reported using infertility services (34). Female subfecundity is the sole cause of reproductive issues in 50% of couples struggling to conceive (35). Fecundity is defined as ones physiological potential to reproduce in a given cycle and is often used interchangeably with the term fertility (36). However, in humans, fertility is the birth rate of a population, which is affected by choice, while fecundity is a reflection of the body's capability to create and sustain a viable offspring. Essentially, fecundity is a reflection of the health of a woman's reproductive system.

In humans, the hypothalamic-pituitary-ovarian axis regulates the female reproductive cycle in a cyclic manner (37-39). Estrogens produced by the ovaries act on the hypothalamus. Gonadotropin releasing hormone (GnRH) is secreted by the hypothalamus and causes the anterior pituitary gland to secrete follicle stimulating hormone (FSH) and lutenizing hormone (LH) (37, 38). FSH and LH then stimulate the ovaries to produce estrogens and the cycle continues (37). The menstrual cycle can be divided into the proliferative and the secretory phases separated by ovulation (38). The estrous cycle in rats is very similar to humans and is divided into four phases (40). The first two phases are proestrus and estrus which correspond to the proliferative phase in humans and the secretory phase matches metestrus and diestrus with ovulation occurring early in metestrus (40). In both humans and rodents the reproduction cycle can be disrupted by metabolic changes, particularly those seen in obesity (9, 41).

Increasing rates of obesity have had a negative impact on female reproductive health or fecundity in humans. One study showed that a 0.1 unit increase in the waist to hip ratio decreased the probability of conception by 30% every menstrual cycle (42). Hassan & Killick describe the relationship between BMI and time to conception as a U shaped curve (Figure 1), wherein underweight women take four times as long to conceive as normal weight women, overweight woman take twice as long and obese women take four times as long to conceive (8). The Collaborative Perinatal Project studied 7327 pregnant women across the United States (7). Regardless of maternal age and menstrual cycle regularity, 75%

of overweight women took 3 months longer than normal weight women to successfully conceive (7). For 75% of obese women it took 9 months longer than normal weight women to become pregnant (7). The majority of studies investigating the consequences of obesity on fecundity only include women who are currently pregnant. Excluding women who have not conceived may underestimate the effects of obesity on reproductive capacity (7).

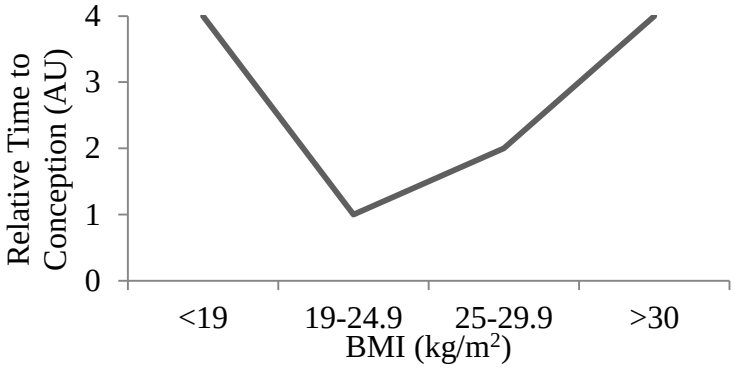

Figure 2.1 Relationship between maternal BMI and relative time to conception (TTC).

Decreased fecundity can also be demonstrated in animal models of overweight and obesity (41). Wu et al. (43) found that mice fed a high fat diet had an approximate anovulation rate of 30% and when ovulation did occur the incidence of fertilization was roughly 20% less than control mice. Mice with diet-induced obesity are characterized by abnormal estrogen cycles and decreased ovulation similar to the symptoms seen in human polycystic ovarian disease (6). Brothers et al. (5) similarly showed impaired fecundity in female mice with diet induced obesity. In contrast, one study performed with the Wistar strain of rats found that inducing obesity did not negatively affect the ability to reproduce (41). This study induced obesity with monosodium glutamate (MSG) instead of a conventional high energy density diet, which may explain the discrepancy. The risks of obesity, however, are not confined to fecundity because when an obese woman successfully conceives, the risk for negative pregnancy outcomes persists.

Studies looking at BMI and pregnancy outcomes have shown significant associations between the risk of adverse pregnancy outcome and high maternal BMI for both natural and assisted reproductive technology (ART) births. One cohort study from the United Kingdom

showed that a maternal BMI $\geq$ 30 kg/m^2 increased the risk of gestational diabetes, hemorrhage, pre-term delivery, caesarean section and high infant birth weight (44). Similarly, a population-based study on a cohort of Danish women found similar results, demonstrating that as maternal BMI increased so did the risk for preeclampsia, gestational diabetes, thrombosis, high birth weight and stillbirths (45). Furthermore, the probability of negative outcomes during ART is increased in overweight and obese women (46, 47). Obese women who undergo *in vitro* fertilization (IVF) have a lower implantation rate and are more likely to have spontaneous abortions after fertilization and implantation has occurred (46, 47).

Hormonal disturbances occur in obesity and can have negative effects on fertility (9). In many studies the relationship between fecundity in obesity and hormonal regulation is complicated by polycystic ovarian syndrome (PCOS) because roughly 30-60% of women with PCOS have obesity (48). PCOS also features hormonal disturbances, specifically hyperandrogenism, resulting in anovulation (48). Independent of PCOS, obesity is associated with impaired regulation of sex hormones, LH, insulin and leptin (9, 48-50). Increased body weight in anovulatory women without PCOS is associated with decreased insulin sensitivity and increased androgen levels (48). Increased insulin concentrations are associated with decreased sex hormone binding globulin (SHBG) levels, which leads to an increase in free androgen concentrations (49-51). Low levels of SHBG and C-reactive protein (CRP), an inflammatory and oxidative stress marker, have been linked to infertility in obese women undergoing ART (49, 50).

Hypersecretion of LH and the subsequent increase in the LH/FSH ratio is associated with an increase in CRP in follicular fluid and reduces oocyte quality (50, 52, 53). Alterations in FSH and LH due to obesity are not consistent across all human and rodent obese models (43, 49, 54). In human work, LH and FSH levels are lower in obese women compared to normal weight women (49). The duration of high fat diet exposure may determine if hormonal changes are elicited in obese rodent models. Mice fed a high fat diet for 4 weeks showed an impairment in fertility, but did not show any significant differences in FSH and LH levels (43), while female rats fed a high fat diet for 120 days exhibited

increased serum insulin, increased LH and progesterone levels resulting in a prolonged estrous cycle (55). A study by Bermejo-Alvarez et al. (56), found that following 12 weeks of high fat feeding roughly half of all female DIO mice were infertile and were characterized by increased serum leptin concentrations.

2.2 Maternal Obesity & Offspring Health

As discussed in Chapter 1, the conveyance of obesity may result from 'programming' or what is sometimes called the Developmental Origins of Health and Disease (DOHaD) hypothesis. The DOHaD hypothesis states that poor nutrition early in life increases offspring susceptibility to morbidity later in life (13). In a classic study performed by Barker & Osmond (13) in the 1980s, the risk of developing heart disease in adulthood was shown to be greatly increased in children who were undernourished *in utero* and during infancy. Similarly, rodent models have found that offspring from high fat diet fed dams were smaller than control (57, 58), which is important given that low birth weight has been correlated with cardiovascular disease, hypertension, T2D and increased risk of becoming obese in adulthood (59). Given that our nutritional environment has progressively shifted to one of overconsumption in recent decades, the focus of developmental programming research has shifted from the effects of undernourishment to that of overnutrition and obesity on offspring health. A cyclic pattern has emerged wherein maternal obesity increases the risk for gestational diabetes (45, 60). In turn gestational diabetes is associated with macrosomia, or excessive birth weight in the infant (60-62). Completing the transgenerational cycle is the evidence that macrosomia subsequently increases the risk for obesity and T2D in adulthood (63, 64).

Maternal obesity has been associated with a wide variety of health problems in children. Recent evidence suggests that maternal diets high in energy, fat, sugar and salt can lead to irreversible liver damage and increase the development of non-alcoholic fatty liver disease in offspring (65). A study in sheep showed that maternal obesity is associated with downregulation of let-7g micro RNA expression in fetal muscle, which may lead to increased intramuscular adiposity during fetal muscle development (66). In children, it was shown that those whose mothers had obesity, hypertension or diabetes during pregnancy had a higher

chance of displaying an intellectual disability in childhood (57, 67, 68). High maternal pre-pregnancy BMI is also associated with higher blood pressure, higher triglycerides and lower HDL in offspring, thereby increasing the risk of cardiovascular disease (69). Most importantly, for the purposes of this project, maternal obesity is associated with obesity risk in offspring (70, 71). *In utero* exposure to maternal hyperphagia (increased food intake), hypertension, fatty liver and abnormal placental function, can impair skeletal muscle function, adipose tissue growth and pancreatic beta cell function in offspring and predispose them to obesity (72).

The literature currently supports three main potential mechanisms that result in fetal programming of obesity and diabetes (72). Firstly, insulin and leptin resistance may disrupt the production and release of neuropeptides from the hypothalamus leading to increased appetite (72). Secondly, it is proposed that overstimulation of the electron transport chain triggered by exposure to a high energy density diet, causes the formation of free radicals. Increased free radical production may upregulate the expression of inflammatory markers that have been linked to the development of insulin resistance (62, 72). Collectively, mechanisms one and two result in insulin resistance. The third mechanism is epigenetics or the "heritable changes in gene expression that are caused by mechanisms other than changes in the underlying DNA sequence", often environmental in nature such as nutrition. DNA methylation, one of the chief ways in which epigenetics manifests, occurs very early in development. Maternal obesity is thought to disrupt the methylation pattern in offspring (72).

2.3 Interventions

Evidence to support weight loss interventions around the time of pregnancy can be controversial. In the Unites States approximately 40-50% of women of child bearing age are trying to lose weight, which is not surprising given the recent increase in obesity rates (73, 74). Roughly 8% of women who are pregnant are trying to lose weight (73, 74) and 35% are trying to maintain weight (73). Exercise and dietary changes were the most commonly reported methods of weight loss used (73). Gestational weight loss (GWL) is more commonly seen in women who are overweight or obese (75-77). In observational studies,

GWL in overweight and obese women is associated with decreased rates of nonelected caesarean section, lower risk of pre-eclampsia, decreased LGA status and fewer neonatal intensive care unit admissions (75, 76). The negative consequences of GWL include premature delivery, SGA infants, decreased placenta weight and decreased umbilical cord length (75-77). The results of one study in obese Sprague-Dawley rats suggests that it may be safe for offspring to reduce energy intake during pregnancy because offspring of dams exposed to 50% caloric restriction during pregnancy, but no maternal weight loss, had similar birth weight to control pups but a decreased percent body fat (78). Despite this there are no experimental trials in humans investigating GWL and the evidence from observational studies about the efficacy and safety of GWL is unclear, so weight loss interventions in pregnancy should be approached with caution (19) and were not included in this work.

2.3.1 Maternal Pre-pregnancy Intervention

Weight-loss during the pre-pregnancy period may represent an ideal time to address overweight and obesity and disrupt the intergenerational cycle of obesity. Callaway et al. (79) found that a lack of pre-pregnancy health initiatives, inaccurate self-assessment of weight, unsuccessful weight loss attempts and poor advice are barriers to pre-pregnancy weight loss interventions. Despite these barriers, there is evidence to support the potential benefits of pre-pregnancy weight loss. The most common types of pre-pregnancy interventions examined to date are bariatric surgery and exercise. A systematic review performed by Forsum & Brantsaeter found that there is currently a lack of published literature investigating the effects of pre-pregnancy dietary induced weight loss on maternal or offspring health (80), which forms part of the rationale for undertaking the present thesis.

2.3.1.1 Fertility & Pregnancy Outcome

Of the studies that have been conducted, weight loss before pregnancy has been associated with improved fertility and pregnancy outcomes. Following an 11% reduction in body weight, overweight women showed decreased LH and the LH/FSH ratio, increased insulin sensitivity and reversal of hyperandrogenism (53). In an ART fertility study in women, Chavarro et al. (81) measured body weight at the time of enrolment in the study and again when women were starting their first ART cycle. A slight decrease in body weight of

approximately 3 kg between enrolment and starting ART resulted in a higher yield of mature oocytes (55, 81), although this was not sufficient to affect clinical outcomes (81). Moran et al. (82) and Galletly et al. (83) found that an increase in the odds of achieving pregnancy in ART following a combination of dietary and exercise treatment or a combination of a support group and exercise. Human studies consistently show that previously infertile women successfully conceive, have uncomplicated pregnancies and have live births following bariatric surgery (84-86). In extremely rare cases maternal bariatric surgery has been linked to fetal vitamin K deficiency that resulted in fatal infant bleeding disorders (87). Bariatric surgery in obese women is associated with a beneficial decrease in the risk of gestational diabetes, gestational hypertension, preeclampsia and macrosomia (86, 88-90), however, the negative risks include shorter gestation, birth defects and SGA infants (88, 89, 91). The ongoing large multi-centered LIFESTYLE study in the Netherlands is a structured lifestyle intervention intended to achieve a weight loss of 5-10% in obese sudfecund women (92). The researchers hypothesize that the intervention would reduce fertility treatment costs, pregnancy complications and improve offspring outcomes (92). The findings of the LIFESTYLE intervention trial could revolutionize fertility care for overweight and obese women and provide concrete recommendations for interrupting the transgenerational cycle of obesity prior to pregnancy.

2.3.1.2 Offspring Programming

As previously stated, maternal obesity is associated with numerous negative health consequences in children. Most research studies investigating maternal obesity treatments look at neonatal outcomes, but only a few studies in humans have investigated the effects of maternal weight loss around the time of pregnancy on the long term health of a child. In children born following bariatric surgery there is a decrease in the incidence of overweight and obesity and an increase in the incidence of normal weight children (90, 93). When specific metabolic characteristics of children born before or following maternal bariatric surgery are compared, children born after maternal surgery had smaller waist and hip girth, lower fasting insulin levels and HOMA-IR, lower blood pressure, lower triglycerides and total cholesterol and decreased CRP than children born before surgery (90, 94). Some of

these differences may be the result of epigenetic modifications. Guenard et al. (94) also compared the methylation patterns between pre and post-surgery children, wherein a total of 5,698 genes had differential methylation patterns.

Animal studies investigating the effects of maternal obesity treatment on long term offspring health show promising results. One study investigating the effects of antioxidant supplementation in dams fed a 'Westernized' diet prior to and during pregnancy found that maternal antioxidant supplementation normalized body fat and glucose tolerance in offspring, and reduced inflammation and oxidative stress compared to offspring from dams consuming a 'western' diet without supplementation (95). Switching obese dams from a high fat diet to a control diet 1 month before mating has beneficial effects on offspring health at 21 and 150 days (96). At 21 days, offspring of dams switched to the control diet showed normalized fat mass, triglycerides, leptin levels and insulin levels compared to those whose mothers remained on the high fat diet (96). By day 150, leptin levels remained normalized to the control group and fat mass and fat cell size although significantly higher than the control offspring, were still significantly lower than the pups from obese high fat fed dams (96). Srinivasan et al. (97) and Gallou-Kabani et al. (98) looked at offspring programming over multiple generations. When dams that were hyperinsulinemic were pair fed to control dams, their progeny showed insulin secretion similar to control progeny (97). Similarly, by reducing maternal fat intake during the periconceptual/gestation/lactation period in mice with high-fat diet (HFD)-induced obesity, almost half of their female progeny showed resistance to a high fat diet (98). The female progeny resistant to the high fat diet displayed normalized energy intake, weight gain, glycemia and insulin levels (98).

Though effective, bariatric surgery is invasive, costly and is only available to women who are extremely obese or have severe weight related health problems in Canada. It is important for all women to achieve a healthy weight going into pregnancy. Villamor & Cnattingius (99) investigated postpartum weight loss and showed that regardless of BMI status at first pregnancy women who gain weight between pregnancies have increased risk of negative pregnancy outcomes including LGA infant, preeclampsia, gestational diabetes and still birth in their second pregnancy. This study illustrates a need for weight management

strategies that are available and safe for all women, regardless of BMI status, during their childbearing years. The present thesis investigates the effectiveness of theoretically safe and non-invasive weight management treatments during the pre-pregnancy period including the prebiotic fibre oligofructose, alone or in combination with the diabetes medication sitagliptin.

2.3.1.3 Oligofructose

Oligofructose (OFS) is a dietary fibre, specifically an oligosaccharide that is similar in structure to inulin, but has a shorter degree of polymerisation (100). Foods that naturally contain oligofructose include onions, artichoke, chicory, garlic, leek, rye, barley, wheat, asparagus and bananas (101). Evidence has accumulated over the past decade in humans and animal models to suggest that oligofructose consumption has many health benefits. Long term consumption in rats is associated with lower body weight and body fat, decreased energy intake and lower plasma triglycerides compared to a standard diet (100). Studies have also linked OFS to improved immune function (102) and bone mineral density (103). One study in cats showed that supplementation of OFS in both a high fat and a control diet decreased protein breakdown for gluconeogenesis (104) which may be beneficial in obesity management particularly during active weight loss. In rats, OFS supplementation in obesity interventions has protective effects on the liver and glucose control and it decreases energy consumption, triglycerides, plasma urea, body weight and hunger compared to groups without OFS (20, 27, 105-108). The beneficial effects of inulin-type fructans are not as conclusive in human RCT, which is likely a result of different experimental designs and dosages (109). Several studies have already shown beneficial programming effects in offspring of dams who consume OFS directly before and/or during pregnancy and lactation (110-112). When exposed to maternal OFS consumption, offspring have lower body weight, lower body fat, higher plasma GLP-1 and higher hepatic GLUT2 at weaning compared to control (110-112).

For the purposes of this study the two most important functions of oligofructose in obesity treatment are its effects on gut microbiota and satiety. The first important function of OFS is that it is a prebiotic fibre. Prebiotic fibres are nondigestible carbohydrates that are fermented in the gut and can selectively stimulate the growth or activation of the

microorganisms in the colon that will result in health benefits for the host (113). OFS is associated with an increase in two specific health-promoting bacteria, Bifidobacteria and *A. muciniphila* (24, 113-115). Bifidobacteria have been shown to have a strong negative correlation with the blood lipopolysaccharide (LPS) (116). LPS contributes to the low-grade inflammation that is associated with obesity and T2D (117). An oral gavage of *A. muciniphila* reduces endotoxemia, fasting glycemia, body weight and adiposity and improves intestinal characteristics in mice on a high fat diet (114). Other important gut microbiota will be discussed in greater detail below. OFS is fermented by bacteria in the gut (113) and short chain fatty acids (SCFA) are produced as by-products (118-120). SCFA production decreases the pH of the colon thereby decreasing the activity of pathogenic bacteria production of toxins (113, 120, 121). Consumption of OFS is associated with an increase in the SCFA receptors, G-protein coupled receptors (GPR) 41 and 43, in the enteroendocrine cells of the small intestine and colon, which are the very cells that produce and secrete the satiety hormones, GLP-1 and PYY (122-124).

The second important function of oligofructose is that it alters secretion of gut peptides. Previous work in both rats and humans demonstrates that OFS treatment is associated with increased plasma peptide YY (PYY) and decreased ghrelin (20, 25, 125). OFS is also associated with increased proglucagon mRNA expression, the precursor to GLP-1, in the proximal colon (108) and in the cecum (25). Increased proglucagon expression as a result of OFS supplementation has a strong positive correlation with GLP-1 content in the colon and in the portal vein (108). In GLP-1 knockout mice, the anti-diabetic effects of dietary supplementation with OFS are absent thereby demonstrating the important role the GLP-1 plays in the actions of OFS (126). Although several studies have shown an increase in GLP-1 with consumption of oligofructose (127) this is not the case for all studies (125, 128). One study by Parnell & Reimer (25) showed a significant increase in proglucagon mRNA levels with prebiotic supplementation, but there was no difference in plasma GLP-1 levels in a genetically obese rodent model.

GLP-1 has a relatively short half-life within the body (129, 130). It is secreted following post-translational modification of proglucagon from L-cells in the distal small

intestine and the proximal colon (22). GLP-1 acts as an incretin, so it stimulates insulin release and promotes β cell proliferation in the pancreas (22, 23, 29). Through the incretin system GLP-1 has also been associated with increased glycogen synthesis in the liver and skeletal muscle, slower gastric emptying and a decrease in appetite (126). GLP-1 levels increase following meals both high in fat and/or high in carbohydrates (23). GLP-1 promotes satiety through the hypothalamus. Given the short half-life of GLP-1 it is not likely that GLP-1 secreted in the colon has a direct effect on the hypothalamus. Instead it has been proposed that GLP-1 stimulates the vagal afferent nerves in the gut which causes GLP-1 production in the brain (131). GLP-1 has important effects in the regulation of metabolism and diabetes. When used to treat newborn intrauterine growth-restricted (IUGR) rats, the GLP-1 analogue Exendin-4 results in normalization of impaired glucose tolerance, β cell mass and gene expression in the liver and pancreas (132, 133) and results in anti-diabetic epigenetic modifications (134).

2.3.1.4 Sitagliptin

As discussed earlier, GLP-1 is broken down within minutes of its release from the L cells (129, 130). GLP-1 is broken down by the ubiquitous enzyme called dipeptidyl peptidase -4 (DPP-4). Inhibition of DPP-4 has anti-diabetic effects because it increases the time that GLP-1 circulates *in vivo* (29, 30, 135). Sitagliptin is one DPP-4 inhibitor that was approved for the treatment of T2D in the United States by the FDA in 2006 and it was approved for use in Canada shortly thereafter. Treatment with sitagliptin in rats and humans has been shown to decrease HbA1c, non-esterified fatty acids (NEFA) and plasma glucose and increase β-cell function, GLP-1 and GIP without weight gain and other adverse effects like hypoglycemia (29, 30, 135-137). In rats, additional treatment effects have been seen, wherein sitagliptin is associated with improved cognitive function (138), increased cardiac function (139), slower progression of hepatic steatosis (140) and reduced damage in kidney injury (141). Sitagliptin is currently not recommended for use during pregnancy or lactation due to a lack of well controlled studies in humans (142, 143). However there is research investigating the T2D drug metformin, which is often used in combination with sitagliptin, during pregnancy for treatment of gestational diabetes. Compared with the standard insulin

treatment for gestation diabetes, metformin appears to be an effective and safe alternative (144). In rodents, maternal metformin treatment is associated with lower inflammation markers at birth, but unfortunately is also associated with increased weight gain and adipose tissue accumulation when offspring are exposed to a high fat diet in adulthood (145, 146).

2.3.1.5 Oligofructose & Sitagliptin

While the combination of OFS and sitagliptin is novel, the concept of combining fibre and anti-diabetic agents is not. In addition to being used in combination with other T2D drugs, sitagliptin has been used in combination with stem cells and polysaccharides (31, 135, 147). One study in Wistar rats showed that in combination with stem cells, sitagliptin is associated with an enhanced lowering of fasting plasma glucose, glucagon and regeneration of β-cells in the pancreas (147). Diabetic rats given the combination of sitagliptin and PolyGlycopleX, a functional fibre, had lower glycated hemoglobin and lower blood glucose following an oral glucose load (31). Metformin has been used in combination with other diabetes drugs and fibre including OFS (21, 148, 149). The combination of metformin and psyllium in humans with diabetes resulted in lower fasting blood glucose, higher HDL cholesterol (lower LDL/HDL ratio) and lower HbA1c than metformin and a placebo (148).

2.3.1.6 Caloric Restriction

Caloric restriction is one of the most common methods used for weight loss (150). In the Behavioral Risk Factor Surveillance System of the U.S. in 2003, it was shown that of the 8% of pregnant women who were trying to lose weight during pregnancy, 50% of them were calorically restricting and 22% of the women trying to maintain their weight were calorically restricting to do so (73). Despite the common use of caloric restriction in weight loss interventions, changes in BMI, fasting insulin and HOMA-IR are inversely correlated with weight regain following this strategy. Furthermore, caloric restriction during pregnancy has been associated with intrauterine growth restriction (IUGR); both are associated with negative programming effects in offspring. Caloric restriction and IUGR have been associated with higher body weight (in males) (151), fasting hyperglycemia, fasting hyperinsulinemia, lower β cell mass, and altered adipose, hepatic and hypothalamic tissue

gene expression (151-153). Not all of the literature regarding caloric restriction during pregnancy and lactation, however, is negative.

Caloric restriction during gestation that continues throughout lactation in rats is associated with lower body weight, lower body fat percent, lower fasting leptin, increased glucose uptake in skeletal muscle, increase oxygen consumption and increased activity in male offspring compared to IUGR males (154, 155). Reynolds et al. (78) found that 50% maternal caloric restriction in rats during pregnancy did not result in negative maternal or fetal outcomes as long as proper nutrient intake was maintained (78). In fact pups born to 30% and 50% caloric restriction dams had significantly less body fat than pups born to control and 15% caloric restriction dams (78). One study investigating 30% caloric restriction in rats during lactation resulted in sex specific benefits in offspring (156), wherein male offspring where more protected against insulin resistance when exposed to a high fat diet and had adipose tissue with better capacity to handle excess energy, while female adipose tissue was more sensitive to leptin signalling (156). One recent study by Nicholas et al. (157) investigating the effects of periconceptual caloric restriction in ewes on offspring hepatic insulin signalling found promising results. Lambs born to ewes exposed to caloric restriction one month prior to and one week following conception showed a reversal in detrimental hepatic insulin signalling compared to lambs from overnourished dams (157).

2.3.2 Gut Microbiota

Intestinal microflora colonization occurs in infancy starting *in utero*. There are several factors that play an important role in determining the final microbiota composition including mode of delivery, location of delivery, prematurity, antibiotic use, breast feeding, hospitalization and the number of siblings (158). One study found evidence to suggest that babies born vaginally at full term, in the home and were breastfed had the highest abundance of beneficial bacteria (158). Recently, a cohort study that followed children delivered by caesarean section were twice as likely to be obese by the time they were three compared to children born vaginally (159). The authors suggested that differences in the gut microbiota may play a role in the increased prevalence of obesity (159).

Increasing evidence shows that gut microbiota have an important role to play in energy harvest and storage in both rodents and humans (160). Scientists suggest that the microbiota can act as a metabolic organ because the interaction between microbiota and their hosts' physiology is so closely linked (160). One way microbiota form a symbiotic relationship with the host is by breaking down foods that are indigestible to their host (160), thereby allowing the host to extract and store more energy from food. In the past, when access to food was limited or only available in a cyclical famine and feast nature, this relationship proved advantageous. However, given the increasing prevalence of obesity and its associated metabolic disorders, there is growing interest in altering gut microbiota in a direction that promotes less energy harvest.

The role of microbiota in obesity development is supported by a number of landmark studies (16, 114, 161, 162). Firmicutes and Bacteroidetes are the two most prominent microbial phyla in the gastrointestinal tract (16, 163, 164). Obesity is associated with more Firmicutes and less Bacteroidetes in both mice and humans (16, 165). When compared to lean mice, obese mice have a 50% decrease in the number of Bacteroidetes (166). Weight loss is correlated with the shift of gut bacteria in favour of Bacteroidetes (166). Additionally, when microbiota from conventionally raised mice were implanted into the gut of germ free mice, a 40% increase in body fat resulted even though the amount of food consumed by the two groups remained the same (160). These findings have not, however been consistent in every case. One comparison of the bacterial groups in obese and non-obese male humans showed no significant difference in the proportion of Bacteroidetes compared to lean controls or after weight loss (28). These discrepancies probably relate to the distinct actions of bacterial subgroups within the two major phyla and other bacterial populations. For this reason it is important to enumerate a broad scope of bacterial groups as will be done in this study.

The microbial groups that will be measured include the Archaea, total bacteria, Bacteroidetes, Firmicutes, Proteobacteria and Actinobacteria. *Methanobrevibacter* is the representative Archaea that will be measured. Within Bacteroidetes, the *Bacteroides/ Prevotella* group will be quantified. Firmicutes will be further divided into the *Clostridium*

leptum, Clostrodium coccoides, Clostrodium cluster I, Clostrodium cluster XI, Roseburia and *Lactobacillus* groups. The *Enterobaceriacea* will be measured for the Proteobacteria group and *Bifidobacterium* for Actinobacteria.

2.4 Summary

It has been demonstrated that the increasing prevalence of obesity in women of childbearing age has many detrimental effects for both mother and child. Overweight and obese women are more likely to struggle with subfecundity and have negative pregnancy outcomes. The periconceptual and *in utero* environments represent crucial developmental periods that have acute and lasting effects on infant outcomes that can modify disease risk in adulthood. Reducing maternal obesity is pivotal to breaking the transgenerational cycle of obesity and preventing chronic disease. While the premise that reducing maternal obesity prior to pregnancy will improve offspring health is logical, well-controlled studies are critically needed to identify strategies that optimize both maternal and offspring outcomes, including dietary and pharmacological interventions.

Chapter Three: **Fecundity& Pregnancy Outcome**

3.1 Introduction

In the year 2008, the prevalence of overweight and obesity in women over 20 years of age in Canada was 55.2% and 23.9% respectively (167, 168). A growing body of evidence suggests that increasing maternal body mass index (BMI) is associated with a decrease in fecundity (7, 169). The Collaborative Perinatal Project in the U.S. found that the probability of conception is decreased by 18% in women with a BMI $\geq$30 kg.m^2 and decreased 8% in women with a BMI between 25-29.9 kg/m^2 compared to women who have a BMI between 18.5-24.9 kg/m^2(7). The reduction in fecundity was independent of menstrual cycle regularity and maternal age (7). Similarly, the Danish National Birth Cohort study found that obese women are more likely to take longer than 1 year to conceive and to undergo infertility treatment than other women (170). In addition to having reduced fecundity, overweight and obese women are more likely to have poor pregnancy outcomes.

Maternal obesity is associated with numerous pregnancy complications (61, 171, 172). The most notable include gestational diabetes, preeclampsia and macrosomia. Macrosomia, which is defined as a birth weight > 4000g in humans, is associated with increased risk of childhood obesity, which in turn is associated with obesity in adulthood (172). At the other end of the birth weight continuum is *in utero* growth restriction which is also associated with adulthood obesity and metabolic disease (173). It is in this context, that changes in body weight prior to pregnancy have been investigated. Diouf et al. (171) demonstrated that weight loss prior to pregnancy in women with a BMI < 25 kg/m^2 was associated with infants that were born small for gestational age. Weight loss prior to pregnancy in overweight women, however, resulted in a trend for reduced maternal risk of gestational diabetes and hypertension, and importantly was not associated with low birth weight in their infants (171). This finding could imply that it is safe to recommend weight loss to overweight and obese women who are contemplating pregnancy. Unfortunately, there is currently a lack of conclusive literature addressing diet-induced weight loss prior to conception in both humans and rodent models (80). As such, the primary aim of this study was to determine if a

combined dietary and pharmacological intervention in the pre-pregnancy period could promote weight loss in obese Sprague-Dawley rats and thereby improve maternal fecundity and pregnancy outcomes.

The dietary component of the intervention was the incorporation of the prebiotic fibre oligofructose (OFS) to the diet of the female rats. OFS has been shown to aid in weight loss and glucose control in part via increases in the levels of the anorexigenic gut hormone glucagon-like peptide 1 (GLP-1) (20, 107). The pharmacological component of the intervention was a recently approved antidiabetic agent, sitagliptin. Sitagliptin is a dipeptidyl peptidase-4 (DPP-4) inhibitor used to treat T2D (30). DPP-4 inhibitors prevent the inactivation of GLP-1, thereby increasing insulin secretion and improving glycemic control (30). By combining the actions of OFS to increase endogenous levels of GLP-1 and actions of sitagliptin to preserve the active form of GLP-1 in circulation, the potential exists for enhanced weight loss and glucose control over either treatment alone.

In addition to its GLP-1 modifying effects, OFS has also been shown to modify the gut microbiota in a manner that reduces inflammation and propensity for obesity (114, 165, 174). Gut microbiota has emerged as an essential component of metabolic regulation in obesity. Some, but not all, research shows that obesity is associated with a microbiota profile that is high in Firmicutes and low in Bacteroidetes (28, 161, 175).The establishment of the gut microbiota appears to be particularly influenced by the early life environment. The profile of the gut microbiota stabilizes around 2 years of age in humans and is affected to a large extent by the mother and the surrounding environment (176).

Given the increase in rates of obesity in women of child-bearing age and the lack of systematic evaluation of interventions to treat obesity in the pre-pregnancy period, our objective was to determine if a combined dietary and pharmacological intervention could promote greater weight loss that would ultimately affect fecundity and pregnancy outcomes in obese female rats. Furthermore, because OFS has proven microbiota modifying effects, we also examined whether the pre-pregnancy treatment would alter gut microbiota profiles in offspring at weaning.

3.2 Methods

3.2.1 Experiment

The research protocol and animal care was approved by the University of Calgary Life and Environmental Science Animal Care Committee. Obesity was induced in 10 week old female Sprague-Dawley (N=120) rats with a high fat/high sucrose (HFS) diet for 14 weeks. The rats with the highest weight gain were then randomized into 1of 6 groups for 8 weeks: 1) Control; 2) 10% OFS (wt/wt); 3) Sitagliptin (10 mg/kg); 4) 10% OFS + Sitagliptin (10mg/kg); 5) Weight matched to group 4 with caloric restriction (CR); 6) Untreated obese (HFS-CON); (n=11-13) in each group). An additional group of rats were fed control diet throughout the entire experiment and served as a lean control group (n=13; Lean-CON). The composition of the experimental diets is provided in Appendix A. The CR, HFS-CON and Lean-CON groups acted as references against which the treatments could be evaluated. At the end of the 8 week intervention the rats were bred with male Sprague-Dawley rats (University of Calgary LESARC, Calgary, AB). Dams in the HFS-CON group (n=12) continued on the HFS diet throughout pregnancy and lactation, while the other dams were fed a control (AIN-93G) diet. The reproductive parameters measured include: 1) fertility, delivery and pregnancy indexes (41); 2) number of live pups at birth (before culling) and pup survival percentage after 2 weeks (177); and 3) sex prevalence (% males/ litter). Dams were considered sterile after 3 unsuccessful breeding attempts (178). Pregnancy outcomes were classified as: 1) normal litter (≥10 pups); 2) small litter (<10 pups); 3) adverse (maternal death or ≥ half of pups dead within 1 week).

3.2.2 Gut Microbiota

Fecal samples were collected from dams at birth and from the offspring at weaning for gut microbiota analysis according to our previous work (25). DNA was extracted from the fecal samples using the MPBiomedicalsFastDNA Spin Kit for Feces (MP Biomedicals, Lachine, QC, Canada). The Thermo Scientific NanoDrop 2000 spectrophotometer was used to quantify DNA concentrations. 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). Samples were run in

duplicate with a final volume of 25 µl containing 0.3 µM primer and 20 ng template gDNA. The specificity of the primers and the limit of detection were determined according to Louie et al. (179). The 16S rRNA gene copies value was calculated according the following webpage: http://cels.uri.edu/gsc/cndna.html using average genome sizes. A total of 10 specific bacterial groups were measured: 1) *Bifidobacterium* (genus in the Actinobacteria phylum); 2) *Bacteroides/ Prevotella* (genus in the Bacteroidetes phylum); 3) *Lactobacillus* (genus in the Firmicutes phylum); 4) *Clostridium leptum* (species in the Firmicutes phylum); 5) *Clostridium coccoides* (species in the Firmicutes phylum); 6) Clostridial cluster I (group in the Clostridia class of the Firmicutes phylum); 7) Clostridial cluster XI (group in the Clostridia class of the Firmicutes phylum); 8) *Roseburia* (genus in the Firmicutes phylum); 9) Enterobacteriaceae (genus in the Proteobacteria phylum); 10) *Methanobrevibacter* (genus in the Archea domain). Total bacteria were also measured. A list of gene specific primers can be found in Appendix B.

3.2.3 Analysis

All data was analysed using IBM SPSS Statistics 19 software. The Fisher's exact test with a Bonferroni adjustment was used to determine if pregnancy outcome differed between maternal treatment groups. Chi Square test was used to determine differences between the pregnancy indexes. A one-way ANOVA and Tukey's *post–hoc* multiple comparisons test was used to determine differences in reproductive parameters, weight outcomes and gut microbiota. Pearson's correlation was used to measure the correlation between maternal fecal microbiota at birth and offspring fecal microbiota at weaning. All outcomes are presented in tables as mean ± SEM, number or percent. The level of significance was set at $P \leq 0.05$.

3.3 Results

3.3.1 Maternal Weight, Reproductive Parameters, & Pregnancy Outcome

Dams in the HFS group had significantly greater weight gain during the treatment phase compared to the sitagliptin and OFS+Sitagliptin groups. Only the OFS+Sitagliptin group, and the CR group (by experimental design), lost weight during the treatment phase, and this was statistically different from all other treatment groups (Table 3.1). At breeding,

the HFS dams weighed significantly more than the Lean-CON, OFS, sitagliptin and CR dams ($P<0.05$). The Control and the OFS+Sitagliptin dams gained the least amount of weight during pregnancy and the CR dams and the HFS dams gained the most (Table 3.1). Weight change during lactation differed significantly between the sitagliptin group and the CR group with the sitagliptin group losing weight and the CR group gaining weight (Table 3.1). Differences in pup weight were only significant for female pups at weaning wherein female pups born to OFS+Sitagliptin dams were significantly heavier than female pups born to Control dams (Table 3.1).

No statistically significant differences were found across the maternal treatment groups for the reproductive parameters examined (Table 3.2). Maternal treatment did however have a significant effect on pregnancy outcome ($p=0.017$). Sitagliptin dams were significantly more likely to have a small litter than Control dams (Table 3.3). Although not significant, no HFS-CON dams had a normal litter and out of all the treatments, HFS-CON dams had the greatest number of adverse outcomes (Table 3.3). Adverse pregnancy outcomes were significantly associated with pup weight at birth and at weaning in both male and female pups. Pups born to dams with adverse pregnancy outcomes weighed significantly less than pups born into small or normal litters (Table 3.4).

Table 3.1 Body Weight Outcomes of Dams and Offspring for the Lean-CON, Control, OFS, Sitagliptin, OFS+Sitagliptin, CR and HFS-CON Maternal Pre-pregnancy Treatment Groups.

	Lean-CON (n=13)	Control (n=13)	OFS (n=12)	Sitagliptin (n=13)	OFS+ Sitagliptin (n=12)	CR (n=11)	HFS-CON (n=12)
Treatment weight change, g	18.5±4.3[ab]	18.9±5.4[ab]	12.1±5.5[b]	13.0±4.0[b]	-6.1±4.4[c]	-8.3±4.1[c]	35.5±6.6[a]
Weight at breeding, g	321±6[b]	348±7[ab]	339±7[b]	347±9[ab]	323±7[b]	324±4[b]	377±14[a]
Pregnancy weight change[1], g	125±7[ac]	85±11[b]	94±10[bc]	117±[7ac]	90±10[b]	132±11[a]	131±5[a]
Lactation weight change, g	15.9±3.7[ab]	10.2±15.5[ab]	10.9±5.1[ab]	-21.9±9[a]	2.3±10.8[ab]	23.8±8.5[b]	0.3±9.2[ab]
Male Birth weight, g	7.2±0.4	6.9±0.3	8.3±0.4	7.4±0.4	6.9±0.6	7±0.3	7.3±0.4
Female Birth weight, g	7.2±0.2[ab]	6.7±0.2[a]	7.7±0.3[ab]	7.3±0.3[ab]	8.0±0.3[b]	6.9±0.2[ab]	7.1±0.3[ab]
Male Weaning weight, g	60±4.9	51.6±7	51.8±5.4	58.7±5.2	53.4±5.2	55.6±4	60.9±5.7
Female Weaning weight, g	56±5	38.5±5.5	50.3±5.5	55.3±5.3	51.7±5.3	55.5±4.4	45.8±7.2

[a,b] Different superscripts indicate significant differences between groups ($P<0.05$).

Table 3.2 Reproductive Parameters Dams in the Lean-CON, Control, OFS, Sitagliptin, OFS+Sitagliptin, CR and HFS-CON Maternal Pre-pregnancy Treatment Groups.

	Lean-CON (n=13)	Control (n=13)	OFS (n=12)	Sitagliptin (n=13)	OFS+ Sitagliptin (n=12)	CR (n=11)	HFS-CON (n=12)
Mated Rats	13/13 (100%)	13/13 (100%)	12/12 (100%)	13/13 (100%)	12/12 (100%)	11/11 (100%)	12/12 (100%)
Fertility Index	13/13 (100%)	13/13 (100%)	10/12 (83.3%)	13/13 (100%)	12/12 (100%)	11/11 (100%)	10/12 (83.3%)
Delivery Index	10/13 (76.9%)	11/13 (84.6%)	9/10 (90.0%)	11/13 (84.6%)	9/12 (75.0%)	10/11 (90.9%)	10/10 (100%)
Pregnancy Index	9/13 (69.2%)	11/13 (84.6%)	8/10 (80.0%)	10/13 (76.9%)	8/12 (66.7%)	9/11 (81.8%)	8/10 (80.0%)
Litter Size, # live at birth	10.3±1.5	8.5±1.5	11.1±1.2	8.33±0.8	6.57±1.1	8.45±1.4	7.14±1.3
Pup Survival,% live at 2 weeks	82.5%	57.4%	64.4%	82.7%	81.0%	71.2%	63.7%
Sex Prevalence, % Males	38.1%	51.6%	40.4%	50.4%	51.1%	36.9%	51.6%

No statistically significant differences exist between maternal treatment groups ($P \geq 0.05$).

Table 3.3. Pregnancy Outcomes for Dams from the Lean-CON, Control, OFS, Sitagliptin, OFS+Sitagliptin, CR and HFS-CON Maternal Treatment Groups.

	Lean-CON (n=13)	Control (n=13)	OFS (n=12)	Sitagliptin (n=13)	OFS+ Sitagliptin (n=12)	CR (n=11)	HFS-CON (n=12)
Normal Litter	4	5	4	2	0	5	0
Small Litter	3^{ab}	0^{a}	1^{ab}	7^{b}	6^{ab}	5^{ab}	5^{ab}
Adverse	3	5	4	2	3	1	6

[a,b] Different superscripts indicate significant differences between groups ($P<0.05$).

Table 3.4. Male and female offspring body weight by normal litter, small litter and adverse pregnancy outcomes. Values are presented as mean±SEM.

	Adverse (n=24)	Small litter (n=27)	Normal litter (n=20)
Male Birth weight, g	6.4 ± 0.4^{a}	7.8 ± 0.2^{b}	7.7 ± 0.2^{b}
Female Birth weight, g	6.3 ± 0.2^{a}	7.6 ± 0.2^{b}	7.5 ± 0.2^{b}
Male Weaning weight, g	39.7 ± 7.4^{a}	60.1 ± 3.1^{b}	56.3 ± 3^{b}
Female Weaning weight, g	27.6 ± 5.9^{a}	57.2 ± 3.2^{b}	55.7 ± 3.1^{b}

[a,b] Different superscripts indicate significant differences between groups ($P<0.05$).

3.3.2 Gut Microbiota

The maternal gut microbiota composition at birth showed significant differences in 3 bacterial groups (Figure 3.1A). *Lactobacillus* spp. was significantly higher in the Lean-CON

group compared to the HFS-CON group (p=0.016). When compared by pregnancy outcome, dams with normal litters had significantly more *Lactobacillus* spp. than dams with small litters and adverse outcomes (p<0.042; Figure 3.2). C. cluster XI also showed significant differences when compared by treatment (p=0.041) and pregnancy outcome. Dams with adverse pregnancy outcomes had significantly higher C. cluster XI than dams with a normal litter (p=0.03; Figure 3.1A). Maternal treatment had a significant effect on the number of *Roseburia* (p=0.008) wherein Control dams had significantly more *Roseburia* than Lean-CON, OFS, OFS+Sitagliptin, CR and HFS-CON (p<0.042).

Male offspring showed significant differences in gut microbiota for *Methanobrevibacter, Bacteroides/Prevotella* and Enterobacteriaceae (Figure 3.1B). The male offspring of HFS-CON dams had significantly higher *Methanobrevibacter* compared to Lean-CON, Control and CR (p<0.048). Offspring of dams that had adverse pregnancy outcomes had significantly greater amounts of *Bacteroides/Prevotella* compared to dams with small litters (p=0.041). Enterobacteriaceae was significantly higher in offspring from dams that had normal litters versus dams that had small litters (p=0.018).

Enterobacteriaceae and C. cluster I differed with maternal treatment group in female offspring (p=0.001; Figure 3.1C). Female pups from dams on the control diet had significantly higher Enterobacteriaceae than all other treatment groups (p<0.014). C. cluster I was significantly higher in CR pups than all other groups (p<0.024).

For dams in the Lean-CON and the CR groups, the Firmicutes:Bacteroidetes ratio was positively correlated with offspring ratios at weaning ($r = 0.762$; $P < 0.028$). In Control dams, maternal C. cluster I was highly correlated with offspring C. cluster I at weaning ($r = 0.917$; $P = 0.01$). The *Roseburia* of OFS treated dams was associated with maternal R*oseburia* at birth. In the sitagliptin and the OFS+Sitagliptin groups, the offspring C. cluster XI was correlated with maternal fecal concentrations ($r = 0.96$; $P < 0.004$). In the sitagliptin alone rats, a significant correlation between maternal and offspring *C. coccoides* numbers at weaning was detected ($r = 0.713$; $P = 0.031$).

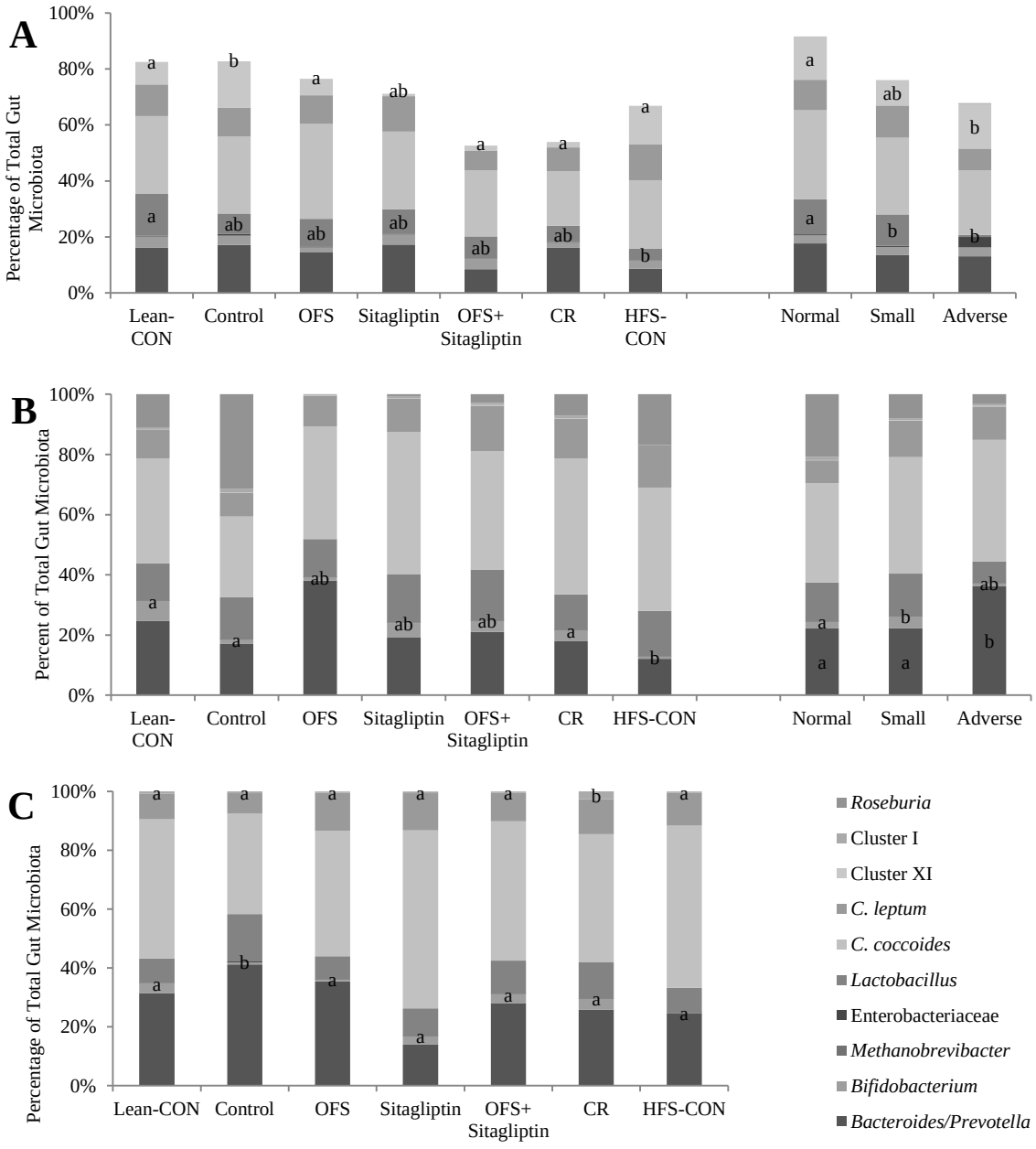

Figure 3.1. Gut microbiota prevalence for Lean-CON, Control, OFS, sitagliptin, OFS+Sitagliptin, CR, and HFS-CON in: A) Dams at birth; B) Male and; C) Female offspring at weaning. Values are presented as percentages of the total gut microbiota, n=8-12 per group. Pregnancy outcome of female offspring was excluded because the adverse group had an n=1. Labelled percentages without a common letter are significantly different (P<0.05).

3.4 Discussion

3.4.1 Reproductive Parameters, Weight & Pregnancy Outcome

Obesity in women of child-bearing age shows a strong association with female infertility (7, 170). Subfecundity in overweight and obese women is thought to result in part from hormonal disturbances and insulin resistance (9, 85). Sex hormones are manufactured in adipose cells and the environment of excessive adiposity leads to endocrine profiles that are characteristic of polycystic ovarian syndrome and anovulation (85). Anovulation in turn is characterised by low progesterone, a disrupted follicle-stimulating hormone/ lutenizing hormone (LH) ratio and high levels of insulin and LH (85). Insulin resistance disrupts the insulin-like growth factor (IGF) system affecting the ovaries and oocyte development and is associated with an increase in circulating LH concentration (9, 53). Reducing body weight, as demonstrated in Butzow et al. (53) resulted in an increase in insulin sensitivity that was inversely related to a decrease in LH.

Given that excessive body fat and insulin resistance play a large role in obesity related-infertility, the objective of this study was to test a pre-pregnancy treatment that combined the effects of OFS and sitagliptin to enhance weight loss in Sprague-Dawley rats resulting in improved maternal fecundity. The present study found that despite significant weight loss in the combined treatment group there were no resulting reproductive benefits. It also appears that pregnancy outcome, independent of maternal weight change, has independent effects on offspring health outcomes. Additionally, treatment with sitagliptin, alone or in combination with OFS, may have a lasting impact on the heritability of offspring microbiota.

Supplementation with OFS is associated with significant weight loss in overweight and obese humans and rats (20, 21, 105). As expected, the actions of OFS and sitagliptin resulted in greater weight loss than either treatment alone in obese female rats in the pregnancy period. Although the OFS+Sitagliptin treatment was associated with weight loss in this study, it was not associated with any improvement in fecundity or pregnancy outcome. The CR group also had significant weight loss, but again there was no association with fecundity. One explanation for this finding could be that despite significant weight loss, the

dams did not lose sufficient weight to result in measureable changes in fecundity. Surgical weight loss has shown positive effects on obesity-related infertility. One study showed that 62.7% of infertile women who underwent bariatric surgery were able to conceive after the surgical intervention (85). Specifically, that study showed that a weight loss of BMI > 5 kg/m^2 was one of the best predictors of becoming pregnant following surgery (85). The results of our study may more closely resemble Chavarro et al. (81), where short term weight loss resulted in an increase in oocytes but did not result in an improvement in fertility treatment outcomes.

Similar to Diouf et al. (171) weight loss prior to pregnancy in obese rats did not result in low birth weight in the offspring. On the other hand, pregnancy outcome appears to be a better predictor of pup weight at birth and weaning than maternal treatment for both male and female pups. Pups born to dams with adverse litter outcomes are significantly smaller than pups born in small or normal litters. The main causes of adverse pregnancy outcomes was maternal death due to pregnancy toxemia, which matches the current literature where hypertensive disorders are associated with low birth weight irrespective of maternal pre-pregnancy BMI (180, 181). The results did not show a direct association between maternal treatment and low birth weight or between maternal treatment and adverse pregnancy outcomes. The association between pregnancy outcomes suggests that pregnancy outcome may have effects on pup birth weight independent of maternal weight status. Many negative health consequences have been associated with low birth weight including enhanced risk of obesity, diabetes and other metabolic related diseases later in life (172, 173). Addressing the causes of low birth weight might prove a good target for reducing the burden of metabolic disease in future generations.

3.4.2 Gut Microbiota

Offspring fecal microbiota was compared to maternal microbiota at birth. A recent study by Koren et al. (182) showed that maternal microbiota is markedly altered between the first trimester and the third trimester of pregnancy. They also showed that the human gut microbial profile does not start to resemble the first trimester maternal microbial profile until 4 years of age in the offspring (182). In fact, one study looking at the relationship in bacteria

between mother and infant found that the highest dissimilarity between maternal and offspring microbiota occurred between late pregnancy and 3 day old newborns (183). Despite the relative instability of the gut microbiome during this phase of development, maternal sitagliptin treatment results in a strong correlation between maternal and offspring C. cluster XI. Sitagliptin acts by inhibiting DPP-4 (30). DPP-4 breaks down GLP-1 through proteolytic inactivation (29). Some members of C. cluster XI have been shown to produce enzymes with proteolytic properties (184). The correlation between maternal and offspring C. cluster XI may be due to alterations in proteolytic activity in the gut in response to DPP-4.

3.5 Limitations

The present study is not without limitations. The age of the dams at the time of breeding (+24 weeks of age) may have precluded an improvement in fecundity that was large enough to detect. Since all of the dams were roughly the same age during breeding, any reductions in fecundity would have been systematic across all of the treatment groups. Given the evidence from human work on drastic surgery-induced weight loss (85) versus short term weight loss (81), the duration of the maternal treatment phase may have been too short. Although we did treat the rats for 8 weeks, a longer period of time might have allowed for sufficient weight loss to result in improved fecundity outcomes.

3.6 Conclusion

The results of this study indicate that small amounts of weight loss prior to pregnancy may have beneficial effects although they may not be enough to reduce the risk of pregnancy complications and negative health outcomes. More research, at the basic and clinical level, is warranted to determine specific weight loss guidelines for women dealing with obesity-related infertility. Regardless of weight status, adverse pregnancy outcomes are associated with significantly lower birth weight in offspring in both male and female pups at both birth and weaning. Continued efforts to reduce low birth weight in all pregnant women regardless of BMI may help to reduce the burden of metabolic disease in the global population. The strong correlation between maternal and offspring microbiota in the treatments involving sitagliptin raises several questions about the role of pharmacology in the programming of the infant gut microbiome that warrant further investigation. Overall the efficacy of the

combined maternal treatment of OFS+Sitagliptin needs to be investigated further. It may have potential to improve maternal pregnancy outcomes and offspring health if sufficient weight loss is achieved.

Chapter Four: **Dietary & Pharmacological Pre-Pregnancy Intervention**

4.1 Introduction

In the year 2009, 59% of Canadians were overweight or obese, and the prevalence continues to rise (61). The high rates of obesity are concerning given that obesity is associated with multiple disease states such as T2D, non-alcoholic fatty liver disorder, cancer, osteoarthritis, cardiovascular disease, hypertension, sleep apnea, decreased reproductive potential and adverse pregnancy outcomes (185, 186). The DOHaD hypothesis suggests that variations in the nutritional environment in the womb and in early life have metabolic consequences that affect the risk of developing chronic disease in adulthood (13). This 'programming' suggests that treatment of maternal obesity prior to conception is of critical importance in the fight against obesity and its related disorders.

There are multiple studies that suggest weight loss prior to pregnancy should improve offspring outcomes in animals and humans (10, 57, 72). However, studies that provide concrete evidence for a significant association between maternal weight loss and improved offspring outcomes are very few. In a French study with 1,756 mother-child pairs, the authors identified a trend showing that voluntary weight loss in the years leading up to pregnancy decreased the incidence of gestational diabetes and hypertension, although it was not significant (171). This same study also showed a trend for normal weight women who lost weight before pregnancy to be more likely to have anemia, but again the finding was not significant. The lack of evidence regarding the effects of weight loss could be due in part to ethical limitations. Studies have shown negative effects of maternal under-nutrition and certain weight loss supplements prior to and during pregnancy (187). Given that there is currently no strong research evidence to show that weight loss for obese pregnant women is safe (19), approval for human intervention studies during pregnancy is a challenge from an ethical standpoint. Nevertheless, evidence for pre-pregnancy interventions from rodent models is promising (111). One study demonstrated percent body fat, fat cell size and leptin concentrations were reduced in male offspring of dams that were switched from a high energy diet to a control diet prior to conception (96). The dams that were switched to control diet showed a trend for decreased body weight, but did not differ significantly from the high

energy dams at breeding (96). At the point of delivery and weaning of the pups at 3 weeks of age, the weight of the dams switched to the control diet was significantly lower than the high energy group (96). A study by Jackson et al. (188) showed that when male offspring were exposed to a maternal high fat diet in utero and then consumed a control diet they had decreased body weight, visceral fat mass and plasma insulin levels at 17 weeks compared to those offspring that consumed a high fat diet postnatal.

Interventions that effectively and safely treat maternal obesity in the period prior to pregnancy are critically needed to reduce the transmission of increased chronic disease risk to offspring (80, 189). Combining a dietary treatment with a pharmacological agent may be more effective than either treatment administered alone. Oligofructose (OFS) is a highly fermentable dietary fibre, called a prebiotic fibre, which has been shown to have positive effects on body fat and satiety hormone concentrations in the blood (20, 22, 25, 107, 190). A recent study in overweight and obese humans showed significantly more weight loss in the OFS group compared to control over a three month intervention period (20). Animal (21, 191) and human studies (20) also show that prebiotic supplementation has a beneficial effect on glucose tolerance, and can beneficially modulate gut microbiota (116). Several mechanisms have been proposed to explain the beneficial effects of prebiotic fibre on glucose tolerance and body weight management, the chief of which is an increase in the secretion of the insulinotropic hormone called glucagon-like peptide 1 (GLP-1) (192).

GLP-1 and peptide YY (PYY) are peptide hormones that are produced and secreted by the intestine and reduce food intake (192). The secretion of GLP-1 and PYY is impaired in T2D and obesity (135, 193) but concentrations can be increased with the consumption of prebiotics (20). The critical role of peptide hormones such as GLP-1 in blood glucose regulation fostered the pharmaceutical development of anti-diabetic agents that promote increased levels of these hormones in the blood, including inhibition of dipeptidyl peptidase-4 (DPP-4). DPP-4 is a ubiquitous enzyme in the body that degrades peptide hormones including GLP-1 (194).

Sitagliptin is a DPP-4 inhibitor that increases the relatively short half-life of gut hormones (192). Sitagliptin is an effective glucose-lowering agent chiefly via its ability to

increase the circulating levels of active GLP-1 in the body. Sitagliptin was approved by the FDA for treatment of T2D in October 2006 (194) and it is now approved in Canada. The development of similar pharmaceutical interventions based on the gut hormone PYY is currently underway. Given that prebiotic fibre increases the endogenous release of GLP-1 and sitagliptin preserves active GLP-1 in the body, their combined actions may be more effective for improving maternal obesity status and glucose tolerance than either alone. Therefore, the hypothesis of this study is that the maternal pre-pregnancy treatment with the combination of OFS and sitagliptin will result in the greatest improvement in offspring metabolic health, including glucose tolerance, gut satiety hormone levels and adiposity in Sprague-Dawley rats.

4.2 Methods

4.2.1 Experiment

All of the research protocols were conducted in accordance with the Guide for Care and Use of Laboratory Animals and were approved by the Life and Environmental Sciences Animal Care Committee at the University of Calgary. Obesity was induced in 10 week old female Sprague-Dawley (N=120) rats with a high fat/high sugar (HFS) diet for 14 weeks. The rats with the greatest weight gain were then randomized into 1of 5 groups for 8 weeks: 1) Control (C; AIN-93M); 2) 10% OFS ; 3) Sitagliptin (10 mg/kg; S); 4) 10% OFS + Sitagliptin (OFS+S); 5) Untreated obese (HFS-CON); (n=12-13 in each group). The AIN-93 and HFS diet compositions have been published previously (21, 195). The OFS diet was prepared by mixing 10g of OFS (Raftilose P95, Quadra Chemicals) with 90g of AIN-93M. The composition was (g/100g): cornstarch (41.9), casein (12.6), dextrinized cornstarch (14), sucrose (9), soybean oil (3.6), Alphacel (4.5), AIN-93 mineral mix (3.2), AIN-93 vitamin mix (0.9), L-cystine (1.6), choline bitartrate (0.23) and OFS (10) (Dyets Inc., Bethlehem, PA, USA). The energy density of the control diet was 3.6 kcal/g, the OFS diet was 3.4 kcal/g and the HFS diet was 4.6 kcal/g. Sitagliptin was administered daily through an oral gavage. A sixth group of rats that did not undergo the obesity induction phase but consumed the control diet *ad libitum* throughout the experiment served as a lean control group (n=13; Lean-CON). The group of HFS-CON dams (n=12) continued on the HFS diet throughout pregnancy and

lactation, while the other dams were fed the control diet (AIN-93G). The HFS-CON and lean groups acted as a reference to determine the effectiveness of the treatment groups. At the end of the 8 week intervention the rats were bred using males from the colony housed at the University of Calgary Life and Environmental Sciences Animal Resource Centre.

At birth litters were culled to 10 pups each with 5 males and 5 females where possible. The pups suckled until 3 weeks of age when they were weaned and one male and one female offspring (N=133) were randomly selected from each litter to participate in the offspring intervention. The rats were weaned onto a control diet (AIN-93G). Body weight was measured weekly and food intake was measured daily. At 11weeks of age, an OGTT was performed to determine glucose tolerance. The rats were then switched to a HFS diet for 6 weeks as a metabolic challenge to unmask any protective or detrimental programming effects. At the end of 6 weeks, a final OGTT was performed to assess glucose, insulin and satiety hormone concentrations (GLP-1, ghrelin, PYY, GIP and leptin). Fecal samples were collected at weaning, 11 weeks and at sacrifice and stored at -80ºC until analysis. Body fat (g) and lean mass (g) were measured using the DXA one day prior to sacrifice. The rats were overanesthetized and the proximal colon was flash frozen.

4.2.2 Blood Glucose & Satiety Hormones

Prior to the OGTT, rats were fasted overnight (12hrs). Blood was collected via a tail nick at 0, 15, 30, 60, 90, 120 minutes and blood glucose was measured immediately using a BD Biosciences, One Touch Blood Glucose Meter. After the fasted blood sample was collected, an oral gavage was used to administer a 2 g/kg glucose load. Additional blood for hormone analysis was collected into tubes containing diprotinin-A (0.034g/L blood: MP Biomedicals, Irvine, CA), Sigma protease inhibitor (1g/L blood: Sigma Aldrich, Oakville, ON, Canada) and Roche Pefabloc (1g/L of blood: Roche, Mississauga, ON, Canada). Active GLP-1, total PYY, total glucose-dependent insulinotropic polypeptide (GIP), active ghrelin, leptin and insulin serum concentrations were measured with the Millipore Rat Gut Hormone Panel kit (Millipore, Billerica, MA, USA). The Millipore Rat Gut Hormone Panel kit has an intra-assay variation < 7%, an inter-assay variation <24% and the spike recovery of the hormones ranges from 84% for GLP-1 to 93% for insulin. Insulin resistance was calculated

using HOMA-IR with the formula: fasting insulin (μU/ml) x fasting glucose (mmol/l) divided by 22.5 (196).

4.2.3 Body Composition

Animals were put under light anesthesia and dual energy x-ray absorptiometry (DXA) was performed (Hologic, ODR 4500: Hologic, Bedford, MA). Hologic QDR software for small animals was used to determine fat mass, lean mass and bone mineral density. An early validation study of the DXA in healthy adults showed that the coefficient of variance for fat mass was 6.4%, bone mineral content was 1.2% and lean tissue mass was 3.1% (197).

4.2.4 mRNA Levels

Trizol Reagent (Invitrogen, Carlsbad, CA) was used to extract the RNA from tissue. The BioRad C1000 Thermal Cycler Real Time PCR instrument amplified the AMPKα-1, GPR41 and GPR43 genes of interest as well as the housekeeping gene β-actin. The detailed protocol has previously been published (198). The primers used to amplify the genes of interest were: 5'–GCCCGACACACCTAGAT-3' (forward) and 5'-TCCAACTGCTTGATTGCTCTAC-3' (reverse) for AMP-activated protein kinase α-1; 5'-ACCCTCTGCTATTCTACTTCTCCTC-3' (forward) and 5'-CCTCCACTGTCTCTTCGGCTC-3' (reverse) for GPR 43; and 5'-TGCTCCTCTTCCTGCCATTCC-3' (forward) and 5'-CGTTCTATGCTCACCGTCATCAG-3' (reverse) for GPR 41.

4.2.5 Gut Microbiota

DNA was extracted from the fecal samples using the MPBiomedicalsFastDNA Spin Kit for Feces (MP Biomedicals, Lachine, QC, Canada). The Thermo Scientific NanoDrop 2000 spectrophotometer was used to quantify DNA concentrations. The BioRadiCycler was utilized to perform the qPCR according to our published protocol (25). The 16S rRNA gene copies value was calculated according the following webpage: http://cels.uri.edu/gsc/cndna.html using average genome sizes. The following bacterial groups were measured: *Bifidobacterium, Bacteroides/ Prevotella, Lactobacillus, Clostridium leptum, Clostridium coccoides,* Clostridial cluster I, Clostridial cluster XI, *Roseburia,* Enterobacteriaceae, *Methanobrevibacter* and total bacteria. The amounts of bacterial DNA

found in the samples are expressed as 16S rRNA gene copies/20 ng DNA. A list of primers can be found in Appendix B.

4.2.6 Analysis

All data was analysed using IBM SPSS statistics software version 19. A two-way ANOVA and Tukey's post–hoc multiple comparisons test were used to determine differences in fasting glucose and hormone levels, lean mass, GPR41 and GPR43 mRNA levels and gut microbiota. Repeated measure ANOVA was used for the timed or longitudinal data including body weight, energy intake and blood glucose from OGTTs. When a significant sex effect was present male and female offspring were analyzed separately. All outcomes are presented in tables as mean ± SEM. The level of significance was set at $P \leq 0.05$.

4.3 Results

Given our objective to test the effects of treatments alone or in combination, only offspring from the four main treatment groups were included in the analysis of the present chapter. The OFS+Sitagliptin maternal pre-pregnancy treatment resulted in significantly more weight loss than all other treatments ($P<0.05$; Table 4.1). Dams from the sitagliptin group gained more weight during pregnancy than control and OFS+Sitagliptin dams ($P<0.05$). There was no significant difference in birth weight of male offspring, but female offspring from the OFS+Sitaglitpin group were significantly heavier than female pups from the Control group at birth ($P=0.016$).

Table 4.1. Weight Change and Litter Statistics of Dams Treated with C, OFS, S or OFS+S Prior to Pregnancy.

	Control	**OFS**	**Sitagliptin**	**OFS+S**
Dam weight change during treatment, g	18.9 ± 5.4^{a}	12.1 ± 5.5^{a}	13.0 ± 4.0^{a}	-6.1 ± 4.4^{b}
Dam weight gain during pregnancy, g	85 ± 11^{a}	94 ± 10^{ab}	117 ± 7^{b}	90 ± 10^{a}
Female pup birth weight, g	6.7 ± 0.2^{a}	7.7 ± 0.3^{ab}	7.3 ± 0.3^{ab}	8.0 ± 0.3^{b}

Male pup birth weight, g	6.9±0.3	8.3±0.4	7.4±0.4	6.9±0.6
Pups, n	8.5±1.5	11.1±1.2	8.3±0.8	6.6±1.1

The values are means ± SEM (n = 11-13 per group). [a,b] Values without a common superscript are significantly different (P<0.05).

4.3.1 Body Weight & Energy Intake

There was no significant difference in body weight of male and female offspring from 3 to 17 weeks of age based on maternal treatment (Figure 4.1). As expected male offspring were significantly heavier than female offspring from 4 weeks of age onward (P<0.0001).

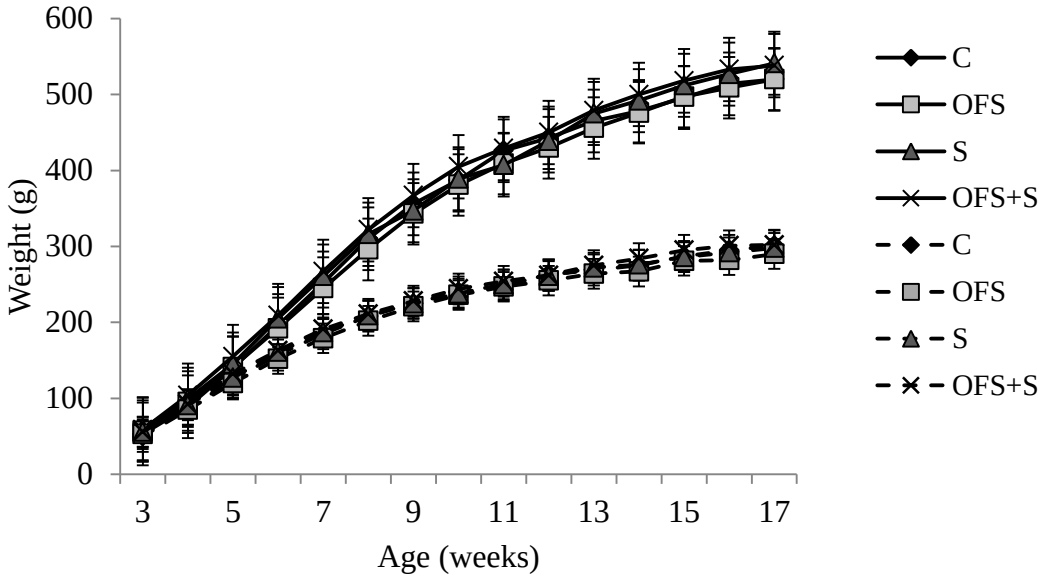

Figure 4.1.Mean offspring body weight from weaning to sacrifice for male and female offspring of dams treated with C, OFS, S or OFS+S prior to pregnancy. Values are mean ± SEM (n=8-12 for males and females). There was a significant effect of week (P<0.0001) and week x sex (P<0.0001). Males are represented with solid lines and females with dashed lines.

Energy intake was significantly affected by the interaction of week, treatment and sex (P=0.002; Figure 2). Male pups from OFS dams consumed significantly more energy than male sitagliptin pups between 6-8 weeks of age (P=0.009). From 9-11 weeks, when the HFS diet was introduced, male OFS pups consumed more energy than all of the other groups

(P=0.0001). Female offspring showed a similar trend. Between 9-11 weeks, female pups from OFS dams had greater energy intake than female pups from sitagliptin dams (P=0.028). From 15-17 weeks, female pups from the Control dams consumed significantly more energy than all other female pups (P≤0.039)

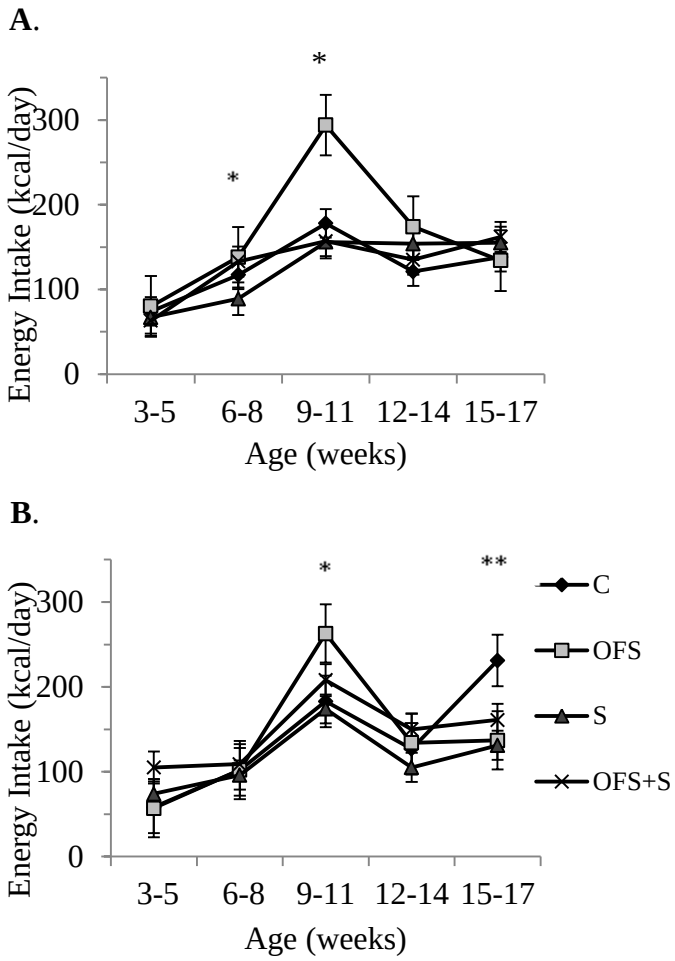

Figure 4.2. Longitudinal energy intake of male (A) and female (B) offspring of dams fed C, OFS, S or OFS+S prior to pregnancy. Values are means ±SEM (n=8-12 for males and females). There was a significant effect of week (P<0.0001) and week x **sex** x **treatment (P = 0.039). The * indicates a significant difference between OFS and all other treatment groups (A at 9-11 weeks) or sitagliptin (A at 6-8 weeks and B at 9-11 weeks) and ** indicates a significant difference between C and all other treatment groups (P<0.05).**

4.3.2 Glucose Tolerance & Satiety Hormones

There was no significant difference in fasting glucose concentration between pups prior to the HFS diet challenge (P=0.42; Table 4.2). Following consumption of the HFS diet for 6 weeks, pups from sitagliptin dams had significantly lower fasting blood glucose than pups from Control dams (P=0.028). At 17 weeks, pups from Control dams had significantly lower fasting leptin than pups from both OFS and OFS+Sitagliptin dams (P≤0.039). Compared to pups from OFS+Sitagliptin dams, OFS pups had significantly less circulating ghrelin at fasting (P=0.023).

Table 4.2.Fasting Blood Glucose and Serum Satiety Hormones in Offspring.

	Sex	Control	OFS	Sitagliptin	OFS+S	Txt	Sex	Txt x Sex
Glucose at 11 weeks, mmol/l	M	6.2±0.2	6.2±0.3	5.9±0.1	6.2±0.3	0.420	0.900	0.632
	F	6.4±0.3	6.2±0.2	6.1±0.2	5.9±0.2			
Glucose at 17 weeks, mmol/l	M	5.8±0.2^{a}	5.8±0.1^{ab}	5.4±0.2^{b}	6.0±0.2^{ab}	0.027	0.231	0.268
	F	6.4±0.3^{a}	5.7±0.2^{ab}	5.6±0.1^{b}	6.0±0.2^{ab}			
Insulin, pg/ml	M	1613±246	1936±356	1274±338	1783±389	0.900	0.066	0.684
	F	1174±321	1149±263	1290±488	1134±350			
Leptin, pg/ml	M	1985±449^{a}	5748±1015^{b}	2183±492^{ab}	5464±1303^{b}	0.003	0.216	0.226
	F	3286±954^{a}	5698±746^{b}	4854±974^{ab}	4669±817^{b}			

Ghrelin, pg/ml	M	146±35.5[ab]	46.9±12.5[a]	55.8±12.8[ab]	195±70.2[b]	0.023	0.019	0.209
	F	188±47.3[ab]	117±16.1[a]	82.2±29.0[ab]	186±25.8[b]			
GIP, pg/ml	M	36.6±7.8	58±16.2	37.9±6.3	55.8±11.9	0.725	0.003	0.843
	F	96.8±30.2	112±38.6	86.5±23.2	79.7±15.7			
PYY, pg/ml	M	26.8±6.1	23.8±5.7	25.8±4.4	39.4±11.9	0.918	0.035	0.520
	F	41.4±6.8	42.1±9.0	45.7±11.1	37.3±8.4			
GLP-1, pg/ml	M	11.5±2.9	9.8±2.5	10.3±2.5	11.2±2.6	0.853	0.236	0.787
	F	6.1±3.3	10.4±5.0	5.4±3.0	9.6±4.2			
HOMA-IR	M	10.2±1.6	12.1±2.4	7.3±2.1	11.7±2.4	0.801	0.096	0.622
	F	7.9±2.4	7.1±1.7	7.8±3.2	7.3±2.2			

Values are means ± SEM (n=8-12 per group). [a,b] Values without common superscript are significantly different within a sex ($P<0.05$). Txt, treatment.

4.3.3 Gastrointestinal Measures

Pups born to dams from the sitagliptin pre-pregnancy group had significantly heavier stomachs then pups born to Control and OFS treated dams ($P\leq0.027$). The small intestine of OFS pups was significantly shorter than pups from sitagliptin dams ($P=0.036$). OFS pups also had a significantly lower small intestine weight than Control pups ($P=0.015$).

Table 4.3. Final Body Fat Percentage, Liver Weight and Intestinal Characteristics in Offspring from the Control, OFS, Sitagliptin and OFS+Sitagliptin Maternal Treatment Groups.

	Sex	Control	OFS	Sitagliptin	OFS+S	Txt	Sex	Txt x Sex
Body fat,%	M	21.8±1.6	18.2±1.7	21.7±2.2	22.1±1.6	0.113	0.654	0.929
	F	22.4±2.3	17.9±1.6	20.1±1.3	21.3±1.5			
Liver weight, g	M	14.7±0.5	15.7±0.9	14.9±0.4	14.5±0.6	0.584	0.0001	0.174
	F	8.8±0.3	7.9±0.3	7.7±0.3	8.1±0.3			
Stomach weight, g	M	1.7±0.05^{a}	1.8±0.06^{a}	2.0±0.06^{b}	1.9±0.07^{ab}	0.006	0.0001	0.614
	F	1.3±0.04^{a}	1.3±0.06^{a}	1.4±0.08^{b}	1.4±0.04^{ab}			
Small intestine length, cm	M	125±2.3^{ab}	122±1.9^{a}	130±1.9^{b}	128±2.7^{ab}	0.046	0.0001	0.570
	F	114±2.3^{ab}	109±2.6^{a}	113±2.2^{b}	113±1.3^{ab}			
Small intestine weight, g	M	6.8±0.3^{a}	6.5±0.3^{b}	6.9±0.3^{ab}	7±0.3^{ab}	0.028	0.0001	0.269
	F	5.8±0.1^{a}	4.9±0.2^{b}	5.2±0.2^{ab}	5.3±0.1^{ab}			
Colon length, cm	M	19.6±0.8	19.2±0.6	20.4±0.4	20.4±0.5	0.206	0.0001	0.940

	F	17.1±0.6	17.4±0.7	18±0.4	18.1±0.8			
Colon weight, g	M	1.2±0.05	1.2±0.08	1.2±0.05	1.2±0.09	0.766	0.0001	0.750
	F	1.0±0.06	0.9±0.06	1.0±0.06	1.0±0.06			

The values are means ± SEM (n=8-12 per group). [a,b]Values without a common superscript are significantly different within a sex ($P<0.05$).

4.3.4 mRNA Levels

Independently, maternal treatment ($P<0.0001$) and offspring sex ($P=0.033$) affected GPR 41 mRNA expression in the proximal colon (Figure 4.3). In the offspring, GPR 41 mRNA levels were significantly higher in pups from OFS+Sitagliptin dams compared to all other maternal treatment groups ($P\leq0.017$). The interaction between maternal treatment and offspring sex ($P=0.001$) affected GPR 43 mRNA in a similar pattern to GPR 41 where male pups from OFS+Sitagliptin dams had higher amounts than all other male offspring ($P<0.0001$). Liver AMP α-1 mRNA levels were significantly affected by the interaction of maternal treatment and offspring sex, where AMPKα1 mRNA levels were significantly higher in male pups from dams in the Control or OFS treatment groups than the sitagliptin or the OFS+Sitagliptin groups ($P<0.0001$; Figure 4.3).

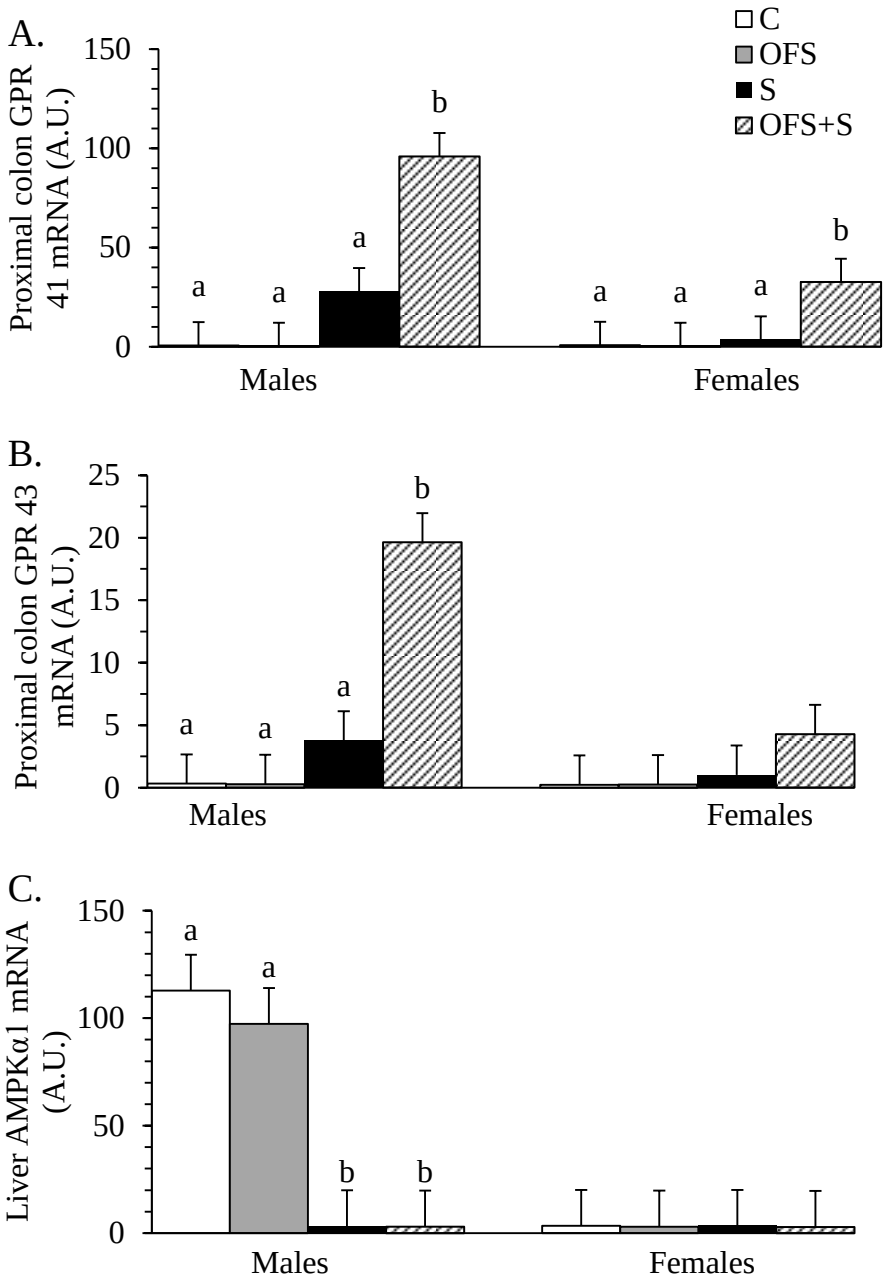

Figure 4.3. Proximal colon (A) GPR41, (B) GPR43 and (C) liver AMPKα1 mRNA levels of 17 week old offspring from dams treated with C: control , OFS: oligofructose, S: sitagliptin or OFS+S: oligofructose + sitagliptin prior to pregnancy. Values are mean ± SEM (n=8-12 per group). [a,b]Values without a common superscript are significantly different within a sex (P<0.05).

4.3.5 Gut Microbiota

Fecal samples were collected when the offspring were 11 weeks of age immediately before the HFS challenge (Table 4.4). Maternal treatment significantly affected Bifidobacteria, Enterobacteriaceae, *Lactobacillus* spp., and *Bacteroides/Prevotella* abundance in the offspring ($P \leq 0.034$). The OFS+Sitagliptin treatment offspring had significantly more Bifidobacteria than the OFS alone offspring ($P=0.005$) and they had significantly more Enterobacteriaceae than the Control offspring ($P<0.0001$). Offspring from sitagliptin dams had significantly greater amounts of *Lactobacillus* spp. and *Bacteroides/Prevotella* than OFS+Sitagliptin offspring ($P \leq 0.04$). Total bacteria, Bifidobacteria, *Methanobrevibacter, C. coccoides* spp., *C. leptum* spp., and the total amount of Firmicutes were all affected by sex, wherein female offspring had greater numbers than male offspring prior to the HFS challenge ($P \leq 0.046$). Male offspring had higher C. cluster XI compared to females ($P<0.0001$). The interaction of maternal treatment and offspring sex affected *Roseburia* ($P=0.015$), wherein male offspring from OFS+Sitagliptin dams had higher numbers than males from all other treatment groups ($P \leq 0.028$).

Table 4.4. Gut Microbiota at 11 Weeks in Offspring of Dams Treated with OFS, Sitagliptin, Both or Neither.

	Sex	Control	OFS	Sitagliptin	OFS+S	Txt	Sex	Txt x Sex
Total bacteria	M	1620403±244563	1114595±106524	1660444±178578	1738225±356568	0.240	0.034	0.712
	F	1892998±90487	1742670±272924	1925955±139351	1892200±233362			
Bacteroides/	M	234669±37116	203351±29614	281381±30843	182619±28306	0.155	0.759	0.554
Prevotella	F	247341±20883	193471±26109	331807±39407	221109±56640			
Bifidobacteria	M	16040±5348^{ab}	6450±2278^{a}	15080±4969^{ab}	32473±13357^{b}	0.005	0.046	0.668
	F	34837±13541^{ab}	5530±1588^{a}	32472±6514^{ab}	51406±16829^{b}			
Methanobrevibacter	M	308±308	218±35.4	271±46.6	295±57.1	0.112	0.0001	0.265
	F	1630±256	1149±154	1154±153	1179±95.3			
Enterobacteriaceae	M	611±164^{a}	498±111^{ab}	1296±319^{ab}	1912±464^{b}	0.0001	0.348	0.391
	F	393±103^{a}	611±137^{ab}	430±58.6^{ab}	1999±659^{b}			
Lactobacillus	M	191678±42536^{ab}	212868±32062^{ab}	297918±53028^{a}	166664±38279^{b}	0.034	0.063	0.849

	F	149401±47424[ab]	88966±40891[ab]	250899±93396[a]	98519±27086[b]			
C. coccoides	M	1381770±270850	1031245±161370	930333±161310	1198862±187440	0.362	0.003	0.608
	F	1715540±264896	1890208±351117	1393081±192480	1528047±142844			
C. leptum	M	133726±49484	95173±23338	132884±31425	101190±30562	0.401	0.0001	0.545
	F	496297±117646	545236±143424	726238±128154	470272±75355			
C. cluster XI	M	1515±242	1595±284	2306±361	2287±326	0.200	0.0001	0.758
	F	608±80.7	698±353	977±499	823±232			
C. cluster I	M	8630±1789	9482±2987	7275±968	16019±3144	0.267	0.170	0.175
	F	13325±2196	10444±2726	14164±1183	12824±3110			
Roseburia	M	25769±5819[a]	19867±5883[a]	25235±7921[a]	1689±437[b]	0.015	0.0001	0.015
	F	188±47.4[a]	170±82.3[a]	268±98.4[a]	220±73.0[a]			
Total Firmicutes	M	2008587±411364	1418849±185502	1456468±206613	1725825±270041	0.600	0.003	0.762
	F	2375359±327680	2183326±374602	2385627±270710	2225463±229753			

Values are mean ± SEM (n=8-12 per group). [a,b] Values without a common superscript are significantly different within a sex ($P<0.05$).

Fecal microbiota was also measured following the HFS intervention when the offspring were 17 weeks of age (Table 4.5). Enterobacteriaceae, *Lactobacillus* spp., and *Roseburia* were significantly affected by maternal treatment ($P \leq 0.047$). Control offspring had significantly more Enterobacteriaceae, *Lactobacillus* spp. and *Roseburia* than sitagliptin pups ($P \leq 0.044$). Control offspring also had more Enterobacteriaceae compared to OFS offspring ($P = 0.024$). C. cluster I was significantly higher in male offspring compared to female offspring ($P < 0.0001$).

Bacteroides/Prevotella, total numbers of Firmicutes, percent Firmicutes, percent Bacteroidetes and the Firmicutes: Bacteroidetes ratio was significantly affected by time. After the 6 week intervention, *Bacteroides/Prevotella* and the percent Bacteroidetes decreased ($P \leq 0.017$), while the total number of Firmicutes, percent Firmicutes and the ratio increased over time ($P \leq 0.001$). The interaction of time and maternal treatment affected Bifidobacteria, Enterobacteriaceae, *Lactobacillus* spp. and C. cluster XI ($P \leq 0.033$) wherein offspring in the Control, sitagliptin and OFS+Sitagliptin groups had a significant decrease in Bifidobacteria following the HFS diet challenge compared to 11 weeks ($P \leq 0.0001$). Enterobacteriaceae increased over time in Control offspring but decreased over time in OFS+Sitaglitpin offspring ($P \leq 0.003$). Independent of treatment group, there was a significant decrease in *Lactobacillus* spp. following the HFS diet challenge ($P \leq 0.026$). OFS offspring had an increased amount of C. cluster XI ($P = 0.001$). Both male and female offspring showed a significant decrease in *Lactobacillus* spp. and C. cluster I and a significant increase in *C. leptum* over time ($P \leq 0.02$). *Methanobrevibacter* and *C. coccoides* increased in male offspring after HFS consumption ($P \leq 0.005$). Female offspring showed a significant decrease in *Methanobrevibacter* over time ($P = 0.006$). The interaction of time with maternal treatment and sex influenced *Roseburia* ($P = 0.024$), wherein the number of *Roseburia* in male offspring from the Control, OFS and sitagliptin maternal treatment groups decreased following the 6 weeks of HFS compared to the number at 11 weeks ($P < 0.0001$).

Table 4.5. Gut Microbiota at 17 Weeks of Age in Offspring from Dams Treated with OFS, Sitagliptin, Both or Neither.

	Sex	Control	OFS	Sitagliptin	OFS+S	Txt	Sex	Txt x Sex
Total bacteria	M	1787771±344180	1794757±133769	1447322±138528	1628238±175857	0.319	0.908	0.982
	F	1770551±213712	1759542±184711	1543517±91733	1643443±64034			
Bacteroides/	M	120514±18711	231077±63560	184036±29585	130359±16722	0.397	0.187	0.557
Prevotella	F	197086±25291	205156±48718	216999±22969	199368±45054			
Bifidobacteria	M	5249±2034	6395±2346	6925±2364	5850±1963	0.60	0.05	0.20
	F	7528±2360	2043±687	1210±266	2854±572			
Methanobrevibacter	M	952±130	1214±154	1111±56.6	1142±254	0.674	0.359	0.765
	F	934±94.4	924±138	1041±123	1124±175			
Enterobacteriaceae	M	2352±699[a]	747±125[b]	1002±280[b]	849±276[ab]	0.027	0.332	0.295
	F	1282±571[a]	807±245[b]	737±214[b]	1133±284[ab]			
Lactobacillus	M	46027±18233[a]	36547±10793[ab]	117786±5270[b]	28509±12605[ab]	0.047	0.881	0.612

	F	66525±25140[a]	21871±7292[ab]	21239±9905[b]	24996±8272[ab]			
C. coccoides	M	1683324±325054	1757458±314986	1567243±258921	1621900±307776	0.633	0.914	0.651
	F	1558111±322738	641704±224349	1431796±161690	2083268±287156			
C. leptum	M	526511±120460	1051284±211450	956347±181053	716135±206073	0.101	0.829	0.589
	F	557425±140724	1038521±159267	746382±174327	1026993±255004			
C. cluster XI	M	4426±1423	6447±4814	1071±263.8	1159±293.3	0.062	0.608	0.963
	F	5227±2237	8191±4211	514±216.9	3005±938.0			
C. cluster I	M	7502±2182	6407±1363	4094±406.3	8061±1519	0.179	0.0001	0.547
	F	986±270.0	3140±2196	399±131.6	1892±1181			
Roseburia	M	561±297[a]	214±52.4[ab]	210±71.8[b]	176±59.2[ab]	0.027	0.878	0.970
	F	511±207[a]	188±52.0[ab]	165±54.6[b]	241±66.6[ab]			
Total Firmicutes	M	2397824±501936	2934353±357975	2561197±411950	2616738±544434	0.56	0.93	0.81
	F	2196877±395813	2713615±245592	2562363±481657	3148471±522263			

Values are mean ± SEM (n=8-12 per group). [a,b] Values without a common superscript are significantly different within a sex ($P<0.05$).

4.4 Discussion

Our hypothesis that OFS+Sitagliptin treated dams would lose significantly more weight than either treatment alone is consistent with our findings and a previous study in Zucker fatty rats using a highly viscous functional fibre in combination with sitagliptin (31). OFS is thought to result in weight loss via GLP-1 and peptide YY (PYY). The mechanism is likely linked in part to the fermentation by-products of OFS utilization by the microbiota, namely short chain fatty acids (SCFA). SCFA bind to the G protein coupled receptors, GPR41 and GPR43, which are associated with production of GLP-1 and PYY (22, 123, 124, 199). Once in circulation, GLP-1 and PYY are able to relay signals centrally that reduce food intake (192). Despite also having potent effects on GLP-1, DPP-4 inhibitors are usually considered to be weight neutral over time (199). Sitagliptin is unique among the DPP-4 inhibitors in that it has been found to result in minor weight loss of 0.9 kg after 6 months in humans (200) making it suitable for use in obesity and the metabolic syndrome.

All of the maternal treatments had distinct effects on offspring. Pre-pregnancy maternal treatment with OFS was associated with an increase in energy intake in offspring when exposed to a HFS diet in early adulthood, increased fasting leptin levels, a significant decrease in small intestine length and weight and a protective effect on Bifidobacteria. Alone, sitagliptin resulted in the lowest fasting blood glucose in offspring following 6 weeks on the HFS diet. In combination, OFS+Sitagliptin resulted in significantly more pre-pregnancy weight loss in the dams, but contrary to our hypothesis this did not result in a difference in body composition, weight or glycemic control in the offspring. However, it was associated with a marked increase in GPR41 and GPR43 expression in the proximal colon of the offspring. Together the results of this study may help to elucidate some of the complexities of fetal programming in maternal pre-pregnancy obesity treatments.

Positive programming effects on offspring metabolism have been found for both male and female offspring following a maternal pre-pregnancy intervention (96, 112, 188). A recent study by Hallam and Reimer (112) showed that female offspring of dams

fed a high fibre diet during pregnancy and lactation had lower body fat percent compared to offspring from Control dams despite having similar body weight. This was not the case in the present study wherein neither maternal fibre intake nor the combined fibre and drug treatment showed a significant association with lower body fat percent or body weight in male or female offspring. The discrepancy in results may be due to the fact that dams in our study were overweight or obese prior to the intervention and that the dams consumed the diets for 8 weeks prior to breeding, so any acute effects of the treatments may have dissipated rapidly. Additionally, the duration of the HFS challenge in the offspring was shorter in our study at 6 weeks versus 8 weeks in Hallam and Reimer (112). Both Zambrano et al. (96) and Jackson et al. (188) found significant benefits from switching dams onto a control diet following a period on a HFS diet. None of our maternal treatments showed a significant association with offspring body fat. This could be related to the timing of our DXA scan which could only be performed at the termination of the study due to restrictions on animal movement between facilities. If any programming effects due to maternal pre-pregnancy treatment did in fact exist early on, they did not persist and/or were of insufficient magnitude to maintain lower body fat following a HFS diet challenge.

The fetal programming effects of sitagliptin have not been studied to date and the fetal programming studies looking at OFS have never looked at mRNA expression of GPR41 and GPR43 (112). GPR41 and GPR43 are expressed in a variety of tissues including the colon, ileum, adipose, skeletal muscle, immune cells and the brain (124, 201-204). A high fat diet had been shown to increase GPR41 and GPR43 in the proximal intestine and adipose tissue of male rodents (205). In the present study the high fat diet challenge did not result in a uniform increase in colon SCFA receptors across all maternal groups. The metabolic effects of GPR43 and GPR 41 are tissue and receptor specific (201). Increased expression of GPR43 in adipose tissue has been associated with lipogenesis, but this was blunted by consumption of prebiotic fibre (205). Conversely, consumption of fermentable indigestible carbohydrates is associated with an increase in GPR43 receptors in the enteroendocrine L-cells in the proximal colon (123). We found

increased GPR41 and GPR43 mRNA levels in the proximal colon of male pups from the OFS+Sitagliptin treated dams. Female pups from OFS+Sitagliptin treated dams also demonstrated a significant increase in GPR41 mRNA levels. Since both OFS and sitagliptin act by increasing the amount of circulating GLP-1 it could imply a role for maternal GLP-1 concentrations in fetal programming of GPR41 and GPR43 expression. This inference is further strengthened by literature indicating a strong positive correlation between the amount of GPR43 immunoreactive enteroendocrine cells and GLP-1-immunoreactive enteroendocrine cells (123).

One possible programming mechanism to explain the effects of the OFS and sitagliptin may involve Gα proteins, a specific category of G proteins. The SCFA receptors and the GLP-1 receptor are both G protein-coupled receptors (GPCR), where the receptor for GLP-1 is a class 2 GPCR (206, 207), while GPR41 and GPR43 are class 1 receptors (208). Despite being from different classes both the GLP-1 receptors and the SCFA receptors couple proteins from the same type of G protein: Gα (208-210). Additionally, a study by Cani et al, (126) demonstrated that GLP-1 receptors were necessary for OFS to have anti-diabetic effects making it advantageous to increase receptor numbers in an obesogenic environment. We propose that dams exposed to a HFS diet and then a period of elevated GLP-1, may program their offspring to resist a HFS diet challenge by increasing the amount of Gα proteins which would increase GLP-1 receptors as well as the SCFA receptors GPR41 and GPR43. More investigation is warranted, given that the expression of GLP-1 receptors was not measured in the present study. Although enhanced anti-diabetic effects of the OFS+Sitagliptin maternal pre-pregnancy treatment were not observed at 17 weeks they may take longer to appear than 6 weeks.

The dominant phyla within the rodent and human gut are Bacteroidetes and Firmicutes (16). Obesity and a high fat diet have, in the majority of cases, been associated with an increase in bacteria from the Firmicutes phyla and a decrease in bacteria from the Bacteroidetes phyla (16, 211, 212). Our 6 week HFS diet challenge resulted in a typical response from offspring with a decrease in *Bacteroides/Prevotella*

and an increase in Firmicutes and the Firmicutes:Bacteroidetes ratio. Within the Firmicutes phyla we found varied and sex specific results. In both male and female offspring *C. leptum* increased, while only male offspring showed an increase in *C. coccoides.* An increase in Clostridales was also seen by de La Serre et al. (213) in rats exposed to a high fat diet. Unlike the other members of the Firmicutes phylum, *Lactobacillus* spp. in the offspring decreased between 11 weeks and 17 weeks. This is in accordance with our recent findings that when exposed to a HFS diet challenge, offspring from dams that consumed a high fibre diet also show a decrease in *Lactobacillus* spp. (MC Hallam and RA Reimer, unpublished results).

Consumption of a high fat diet has been previously associated with a significant decrease in Bifidobacteria (27, 212). The combined OFS+Sitagliptin group had significantly higher Bifidobacteria than the OFS alone treatment at 11 weeks, but along with the Control and sitagliptin groups, OFS+Sitagliptin decreased following 6 weeks of HFS diet as previously seen in the literature. However, pups from the OFS group maintained their abundance of Bifidobacteria spp. despite being exposed to the same HFS diet challenge. Studies investigating the effects of prebiotics on gut microbiota have found that consumption of OFS is associated with an increase in Bifidobacteria and that when OFS is combined with a HF diet it restores Bifidobacteria to control amounts (24). Although the present study did not show an overall increase in Bifidobacteria in offspring from dams that consumed OFS, we did show that maternal treatment with OFS prior to pregnancy has protective effects on offspring Bifidobacteria following a 6 week HFS diet challenge. Bifidobacteria has been shown to have protective effects on blood glucose and inflammation in a high energy environments (116).

4.5 Limitations

The present study has three main limitations. Firstly, the timing and duration of our HFS intervention may have influenced the results. The HFS diet challenge in Hallam and Reimer (112) was 2 weeks longer than the present study. Secondly, the pups in the Hallam study (112) were exposed to the HFS diet at an older age and the significant differences in body weight did not appear until 22 weeks of age. Thirdly, the addition of

body composition measures and satiety hormone concentrations prior to the HFS challenge would have facilitated comparison of any changes that occurred during the 6 week HFS challenge and may have been useful in determining differences in the effectiveness of the maternal pre-pregnancy treatments in offspring prior to any high fat metabolic challenge.

4.6 Conclusion

Taken together the results suggest that a combined pre-pregnancy maternal obesity treatment of OFS+Sitagliptin does not improve glucose tolerance, satiety hormone concentrations and adiposity in offspring to a greater extent than either treatment alone. However, the maternal OFS+Sitagliptin treatment does result in programming of distinct features of gut physiology that may have a role in the prevention of T2D. Further research is called for to determine the tissue and gender specific link between GPR41, GPR43 and GLP-1 receptors. The protective effects demonstrated by pre-pregnancy maternal OFS treatment suggest it may have potential to reduce the metabolic burden on offspring health in an obesogenic environment. Further research in this area should target the OFS dose and the safety of OFS consumption during pregnancy and lactation. Overall some potential improvements in offspring health were seen and seem to be centred on GLP-1 and Bifidobacteria as a result of the OFS and the OFS+Sitagliptin maternal pre-pregnancy treatments.

Chapter Five: **Caloric Restriction**

5.1 Introduction

Nearly a quarter of all adults worldwide are overweight or obese (1) suggesting that the global environment is shifting from one of undernutrition to that of overnutrition. As obesity rates have increased worldwide so has the number of women that are entering pregnancy with a BMI > 25 kg/m^2 and a transgenerational cycle of obesity and diabetes has emerged (214). Maternal obesity and/or diabetes is associated with fetal and postnatal overnutrition leading to childhood obesity and early onset T2D (214). In the current nutritional environment, overnutrition often continues throughout life resulting in adult obesity, T2D and metabolic syndrome (70, 214). Disrupting this cycle is crucial to slowing the obesity epidemic.

One way to disrupt the transgenerational cycle may be to reverse or alter fetal obesity programming. Programming occurs during periods of plasticity or rapid growth, chiefly *in utero* and in infancy (12). The present study utilized a maternal weight loss intervention prior to pregnancy in an attempt to reduce the transmission of obesity risk to offspring. The pre-conception period was selected because interventions during lactation and post weaning have limited success (156, 215) and interventions during pregnancy are controversial with regard to the metabolic costs for offspring (151). There is some evidence that it may be safe to use weight loss interventions in overweight and obese mothers in rodents during pregnancy (78) and humans prior to pregnancy (171) as long as proper nutrient intake is maintained. However, the evidence to support weight loss during pregnancy in obese women comes from observational studies and needs to be investigated by more rigorous experimental designs to ensure it is safe (19). For this reason the present study intervened prior to conception in an attempt to maximize the potential benefits and reduce any potential risks associated with malnourishment in offspring. Maternal weight loss prior to conception may prevent fetal programming of obesity in offspring and increase the odds of offspring escaping the cyclic pattern of obesity.

Dietary restriction is a common component of all weight loss interventions (150), but can be difficult to adhere to and is often associated with weight regain (32). Furthermore, intentional caloric restriction prior to conception may not be practical because approximately half of pregnancies are not planned (216). Maternal pre-pregnancy interventions, however, may not need to be as severe as caloric restriction to impart beneficial effects on offspring health. Improving maternal diet quality, by reducing excess energy, prior to conception may be sufficient and more practical to reverse some of the negative effects of high maternal BMI (96, 217). One study in rats showed that offspring born to dams switched from a high energy diet to a control diet one month prior to conception had lower fat mass, triglycerides, leptin, insulin and insulin resistance index compared to offspring from dams who remained on the high energy diet (96). Our hypothesis is that calorically restricting obese dams for eight weeks prior to pregnancy will result in greater improvements in offspring body composition, glucose tolerance and gut satiety hormones compared to offspring from obese dams that consume a control diet before pregnancy.

Distinct microbiota profiles in the human and rodent gut have been linked to obesity (16). The gut microbiota has been shown to affect the pathogenesis and persistence of obesity and metabolic disease through energy harvest and intestinal inflammation (27, 211). Given the importance of gut microbiota for host metabolic response, a detailed investigation of how maternal caloric restriction affects the microbiome of offspring is also included in the present study.

5.2 Methods

5.2.1 Experiment

The University of Calgary Animal Care Committee approved all of the research protocols that conformed to the Guide for the Care and Use of Laboratory Animals. Obesity was induced in 10 week old female Sprague-Dawley rats with a high fat/high sucrose (HFS) diet for 14 weeks. Rats in the top 50^{th} percentile for weight gain were selected for the intervention study. The obese rats were randomized into 1 of 3 groups for 8 weeks: 1) Control (AIN-93); 2) 30% Caloric Restriction (CR); 3) Untreated obese

(HFS-CON); (n=12-13 in each group). A fourth groups of rats that did not undergo the obesity induction and consumed control diet throughout the entire study (Lean-CON) were also included (n=13). The specific diet compositions can be found in Appendix A. At the end of the 8 week intervention all rats where bred using males from the colony housed at the University of Calgary LESARC. The group of the HFS dams (n=12) continued on the HFS diet throughout pregnancy and lactation, while the other dams were fed the control diet (AIN-93G).

At birth the litters were culled to 10 pups each with 5 males and 5 females were possible. The pups suckled until 3 weeks of age when they were weaned and one male and one female offspring (n=182) were randomly selected from each litter to participate in the offspring intervention. The rats were weaned onto a control (AIN-93G) diet. Body weight was measured weekly and food intake was measured daily. At 11weeks of age, an OGTT was performed to determine glucose tolerance. The rats were then switched to a HFS diet for 6 weeks. At the end of 6 weeks, a final OGTT was performed to assess glucose, insulin and satiety hormone levels (GLP-1, ghrelin, PYY, GIP and leptin). Fecal samples were collected at weaning, 11 weeks and at sacrifice and stored at -80ºC until analysis. Body fat (g) and lean mass (g) were measured using DXA one day prior to sacrifice. The rats were overanesthetized and the proximal colon was flash frozen. Real-time PCR was used to determine AMPK, GPR41 and GPR43 (short-chain fatty acid receptors) mRNA levels in the liver or colon as appropriate.

5.2.2 Blood Glucose & Satiety Hormones

The OGTT was performed after an overnight fast of 12hrs. Blood samples were collected from a tail nick at 0, 15, 30, 60, 90, 120 minutes and glucose concentrations measured immediately using a BD Biosciences, One Touch Blood Glucose Meter. After collecting the fasted blood sample, an oral gavage was used to administer a 2 g/kg glucose load. Blood for satiety hormone analysis was collected into tubes containing diprotinin-A (0.034g/L blood: MP Biomedicals, Irvine, CA), Sigma protease inhibitor (1g/L blood: Sigma Aldrich, Oakville, ON, Canada) and Roche Pefabloc (1g/L of blood: Roche, Mississauga, ON, Canada). Active GLP-1, total PYY, total GIP, active ghrelin,

leptin and insulin serum concentrations were measured with the Milliplex Rat Gut Hormone Panel kit (Millipore, Billerica, MA, USA).

5.2.3 Body Composition

Animals were placed under light anesthesia and a dual energy x-ray absorptiometry (DXA) scan was performed (Hologic, ODR 4500: Hologic, Bedford, MA). Hologic QDR software for small animals was used to determine lean and fat mass.

5.2.4 mRNA Levels

Trizol Reagent (Invitrogen, Carlsbad, CA) was used to extract the RNA from tissue. The BioRadiCycler Real Time PCR instrument amplified the AMP α-1, GPR41 and GPR43 genes of interest and the reference gene, β-actin, according to our previously published protocol (198). The primers used to amplify the genes of interest were presented in the previous chapter.

5.2.5 Gut Microbiota

DNA was extracted from the fecal samples using the MPBiomedicalsFastDNA Spin Kit for Feces (MP Biomedicals, Lachine, QC, Canada). The Thermo Scientific NanoDrop 2000 spectrophotometer was used to quantify DNA concentrations. The BioRadiCycler was utilized to perform the qPCR according to our published protocol (25). The 16S rRNA gene copies value was calculated according the following webpage: http://cels.uri.edu/gsc/cndna.html using average genome sizes. The following bacterial groups were measured: *Bifidobacterium, Bacteroides/ Prevotella, Lactobacillus, Clostridium leptum, Clostridium coccoides,* Clostridial cluster I, Clostridial cluster XI, *Roseburia,* Enterobacteriaceae, *Methanobrevibacter* and total bacteria. The amounts of bacterial DNA found in the samples are expressed as 16S rRNA gene copies/20 ng DNA.

5.2.6 Statistical Analysis

All data was analysed using SPSS version 19 software. A one-way ANOVA and Tukey's post–hoc multiple comparisons test were used to determine differences in fasting glucose and hormone levels, lean mass, GPR41 and GPR43 mRNA levels and gut microbiota. A repeated measure ANOVA was used for the timed or longitudinal data

including body weight, energy intake and blood glucose concentrations from OGTTs. All outcomes are presented as mean ± SEM. The level of significance was set at $P \leq 0.05$.

5.3 Results

During the pre-pregnancy treatment, dams from the CR group lost significantly more weight than the HFS-CON group ($P<0.05$; Table 5.1). Dams from the Control group had significantly less gestational weight gain compared to all other maternal treatment groups ($P<0.05$). There were no significant differences in pup weight or litter size between the pre-pregnancy treatment groups ($P \geq 0.05$).

Table 5.1. Weight Change and Litter Statistics of Dams Treated with Lean-CON, C, CR or HFS-CON Prior to Pregnancy.

	LEAN-CON	**Control**	**CR**	**HFS-CON**
Maternal weight change during treatment, g	18.5 ± 4.3^{ab}	18.9 ± 5.4^{ab}	-8.3 ± 4.1^{b}	35.5 ± 6.6^{a}
Maternal weight gain during pregnancy, g	125 ± 7^{a}	85 ± 11^{b}	132 ± 11^{a}	131 ± 5^{a}
Female pup birth weight, g	7.2±0.2	6.7±0.2	6.9±0.2	7.1±0.3
Male pup birth weight, g	7.2±0.4	6.9±0.3	7.0±0.3	7.3±0.4
Pups, n	10.3±1.5	8.5±1.5	8.5±1.4	7.1±1.3

The values are means ± SEM (n = 11-13 per group). [a,b]Values without a common superscript are significantly different ($P<0.05$).

5.3.1 Body Weight & Body Composition

The body weight of male and female offspring from weaning to 17 weeks of age is shown in Figure 5.1. There was no independent effect of maternal treatment on body weight, but the interaction of maternal treatment, time and sex significantly affected body weight ($P=0.011$). As expected, body weight increased with increasing age and male

pups were significantly heavier than female pups starting at 5 weeks for Lean-CON, CR and HFS-CON ($P \leq 0.044$) and starting at 6 weeks for Control ($P \leq 0.001$).

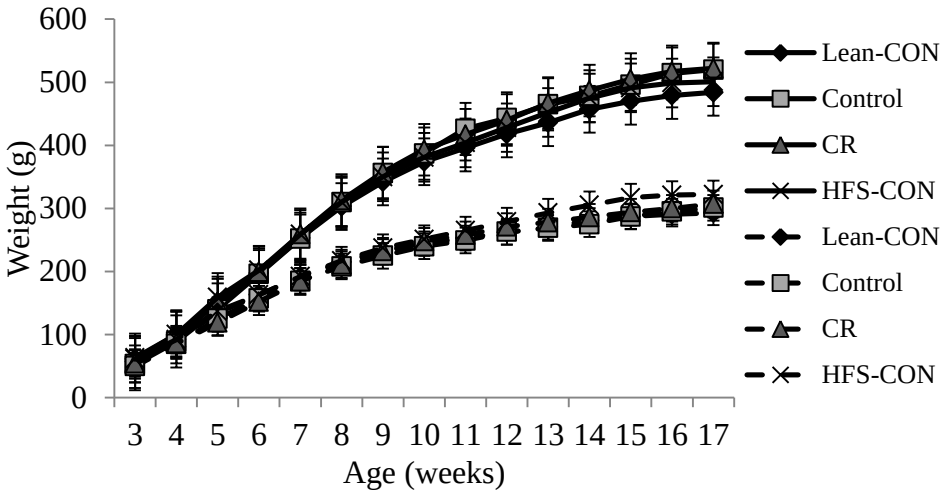

Figure 5.1. Offspring body weight from weaning to sacrifice for male and female offspring of dams treated with Lean-CON, C, CR or HFS-CON prior to pregnancy. Values are mean ± SEM (n=8-12 for males and females). There was significant effect of week ($P<0.0001$) and week x sex ($P<0.0001$). Males are represented with solid lines and females with dashed lines.

Maternal treatment and sex had no effect on offspring energy intake; however, there was a significant effect of week ($P<0.001$) wherein energy intake increased as the rats got older.

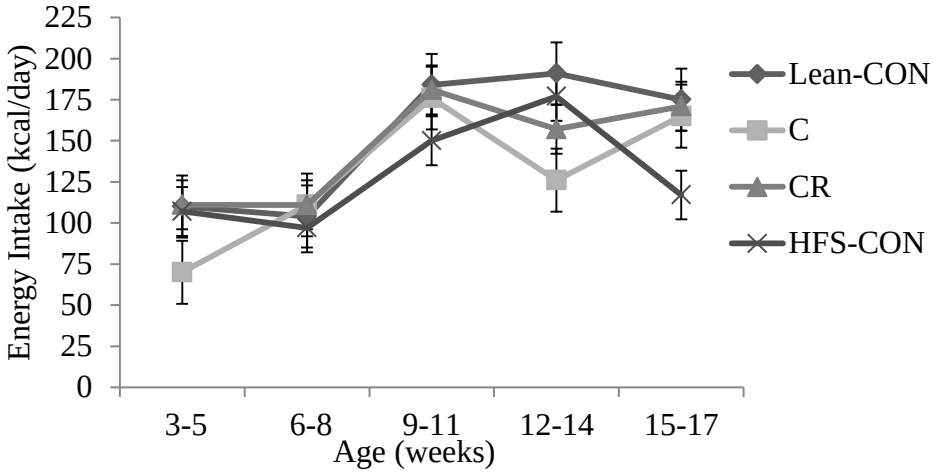

Figure 5.2. Energy intake in offspring from the Lean-CON, C, CR and HFS-CON maternal treatment groups. Values are mean ± SEM (n=8-12). There was a significant effect of week (P<0.0001).

Maternal treatment significantly affected percent body fat in offspring (P=0.029; Figure 5.2). The pups from CR dams had significantly higher body fat percent than pups from dams never exposed to a HFS diet (Lean-CON) (P=0.041). Expressed in grams, final fat mass showed the same pattern, where the Lean-CON group had lower fat mass than CR pups (p=0.047; Figure 5.2). Fat mass was also higher in males than in females (P<0.0001). There was no statistical difference between maternal treatment groups in regards to lean mass (Figure 5.2).

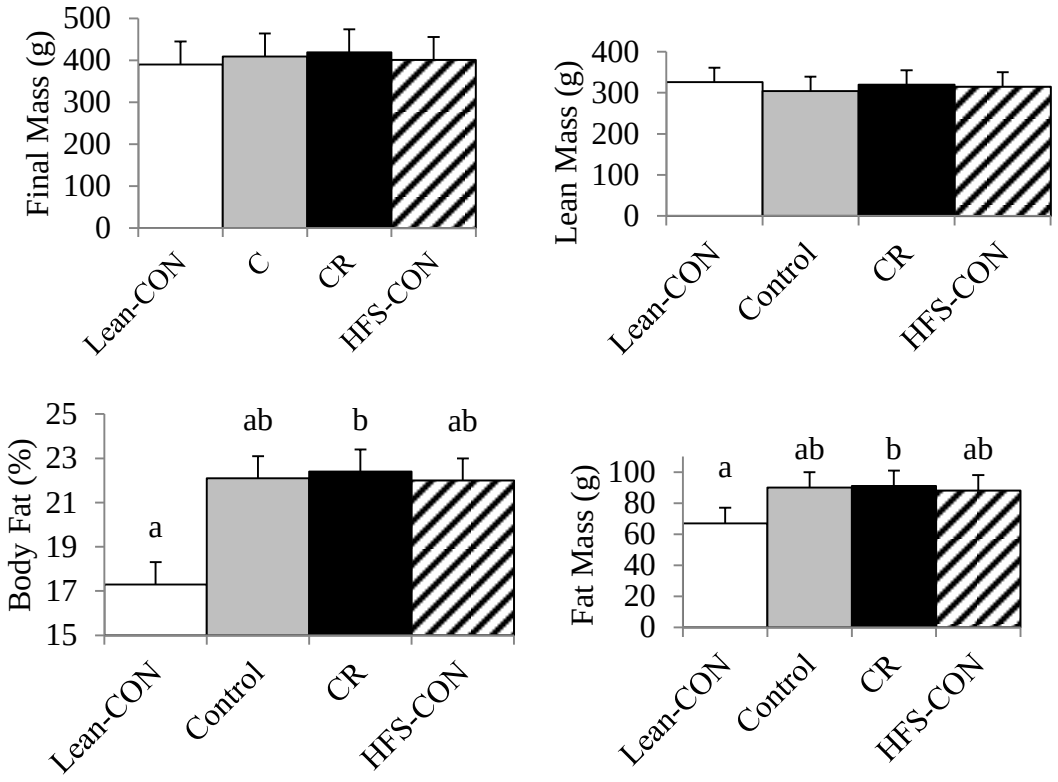

Figure 5.3. Final mass , body fat percent, fat mass and lean mass in offspring from the Lean-CON, C, CR and HFS-CON maternal treatment groups. Values are mean

± SEM (n=8-12). There was a significant maternal treatment effect for body fat % (P=0.029) and fat mass (P=0.044).

There was a significant effect of sex on liver, stomach, small intestine and colon weight ($P<0.0001$; Table 5.2) where males had higher values than females. Maternal treatment affected stomach and small intestine weight ($P\leq0.034$; Table 5.2) wherein offspring from Lean-CON dams had lower stomach weight than CR offspring (P=0.009) and lower small intestine weight than Control offspring (P=0.03). The interaction of sex and maternal treatment affected cecum weight (P=0.017), wherein female Control offspring had significantly higher cecum weight than female CR offspring (P=0.015).

Table 5.2. Final Liver, Stomach, Cecum and Intestine Weight in Offspring from the Lean-CON, C, CR, and HFS-CON Maternal Treatment Groups.

	Sex	Lean-CON	Control	CR	HFS-CON	Txt	Sex	Txt x Sex
Liver weight, g	M	14.4±0.5	14.7±0.5	14.6±0.5	14.8±0.6	0.87	0.0001	0.98
	F	8.4±0.4	8.8±0.3	8.5±0.3	8.5±0.3			
Stomach weight, g	M	1.7±0.08^{a}	1.7±0.06^{ab}	1.9±0.07^{b}	1.8±0.07^{ab}	0.024	0.0001	0.93
	F	1.2±0.05^{a}	1.3±0.04^{ab}	1.4±0.05^{b}	1.3±0.05^{ab}			
Small intestine weight, g	M	6.5±0.2^{a}	6.8±0.3^{b}	7.0±0.2^{ab}	6.5±0.2^{ab}	0.034	0.0001	0.46
	F	5.3±0.1^{a}	5.8±0.1^{b}	5.5±0.2^{ab}	5.4±0.1^{ab}			
Cecum weight, g	M	0.74±0.04^{a}	0.68±0.05^{a}	0.75±0.03^{a}	0.74±0.04^{a}	0.57	0.0001	0.017
	F	0.52±0.02^{ab}	0.66±0.06^{a}	0.49±0.02^{b}	0.54±0.03^{ab}			
Colon weight, g	M	1.2±0.07	1.2±0.05	1.2±0.04	1.2±0.06	0.56	0.0001	1.0
	F	1.0±0.04	1.0±0.06	1.0±0.03	0.9±0.04			

The values are means ± SEM (n=8-12 per group).^{a,b}Values without a common superscript are significantly different within a sex ($P<0.05$).

5.3.2 Blood Glucose & Satiety Hormones

Offspring sex significantly affected fasting glucose, insulin, ghrelin, GIP and HOMA-IR ($P \leq 0.045$; Table 5.3). Female offspring had higher blood glucose than males at the 11 week baseline OGTT ($P=0.014$) and at the final OGTT following the HFS diet challenge ($P=0.018$). Female fasting insulin was significantly lower than males ($P=0.005$), while female fasting ghrelin and GIP was higher than males ($P \leq 0.045$). The HOMA-IR of male offspring was higher than female offspring ($P=0.009$; Table 5.3). Leptin, ghrelin and GIP were all significantly affected by maternal treatment ($P \leq 0.03$; Table 5.3), where the offspring from the Lean-CON group had significantly higher fasting ghrelin and GIP than the HFS offspring ($P \leq 0.046$) and had more leptin than the Control offspring ($P=0.006$). No significant effects were seen in GLP-1 or PYY (Table 5.3).

Table 5.3. Fasting Blood Glucose and Serum Satiety Hormones in Offspring.

	Sex	Lean-CON	Control	CR	HFS-CON	Txt	Sex	Txt x Sex
Glucose at 11 weeks, mmol/l	M	5.7±0.2	6.2±0.2	6.1±0.2	5.6±0.1	0.24	0.018	0.24
	F	6.6±0.3	6.4±0.3	6.3±0.2	5.9±0.2			
Glucose at 17 weeks, mmol/l	M	6.0±0.2	5.8±0.2	5.9±0.2	5.7±0.3	0.78	0.014	0.92
	F	6.4±0.3	6.4±0.3	6.1±0.3	6.2±0.3			
Insulin, pg/ml	M	2222±854	1613±246	1765±270	1125±205	0.41	0.005	0.50
	F	895±185	1174±321	840±109	788±94.4			
Leptin, pg/ml	M	5450±1122[a]	1985±449[b]	4368±860[ab]	3782±814[ab]	0.01	0.46	0.88
	F	5779±843[a]	3286±954[b]	4637±1001[ab]	3706±665[ab]			
Ghrelin, pg/ml	M	178±30.0[a]	146±35.5[ab]	181±32.6[ab]	86±29.6[b]	0.03	0.001	0.72
	F	299±34.5[a]	188±47.3[ab]	265±47.7[ab]	194±31.2[b]			
GIP, pg/ml	M	106±31.3[a]	36.6±7.81[ab]	42±9.14[ab]	43±14.4[b]	0.017	0.045	0.55

	F	124±30.2[a]	96.8±30.2[ab]	90±24.7[ab]	46±13.1[b]			
PYY, pg/ml	M	29.5±2.8	26.8±6.1	32.4±4.4	28.1±5.8	0.72	0.59	0.55
	F	42.1±23	41.4±6.8	23.3±5.6	25.0±7.0			
GLP-1, pg/ml	M	11.4±3.0	11.5±2.9	9.5±2.9	10.5±3.3	0.42	0.89	0.49
	F	20.1±18	6.1±3.3	6.6±6.2	12.4±9.0			
HOMA-IR	M	15.8±6.2	10.2±1.6	11.3±1.9	6.7±1.4	0.26	0.009	0.33
	F	6.2±1.4	7.9±2.4	5.6±0.9	5.4±0.8			

Values are means ± SEM (n=8-12 per group).[a,b]Values without common superscript are significantly different between within a sex ($P<0.05$).

5.3.3 mRNA Levels

GPR41, GPR43 and AMPKα1 expression were all affected by the interaction of offspring sex and maternal treatment ($P\leq0.001$; Figure 5.4). In male offspring, the maternal CR group had higher GPR41 and GPR43 mRNA levels than all other groups ($P\leq0.013$). For male offspring, the Lean-CON and Control group had significantly higher AMPKα1 mRNA levels than the CR and the HFS-CON groups ($P\leq0.0001$).

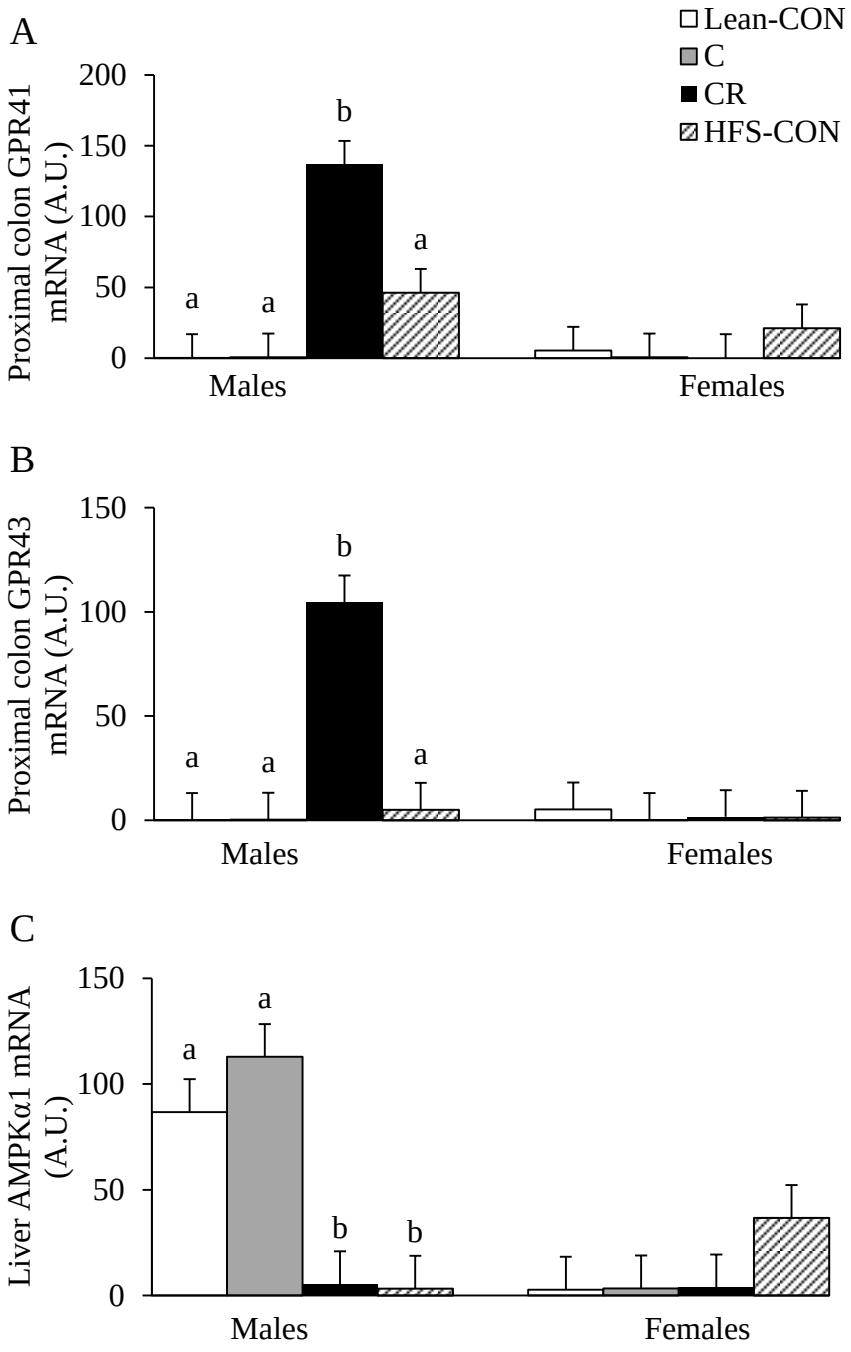

Figure 5.4.Proximal colon (A) GPR41, (B) GPR43 and (C) hepatic AMPKα1 mRNA levels of 17 week old offspring from dams treated with Lean-CON, C, CR or HFS-CON prior to pregnancy. Values are mean ± SEM (n=8-12 per group). [a,b]Values without a common superscript are significantly different within a sex (P<0.05).

5.3.4 Gut Microbiota

Fecal samples for gut microbiota analysis were collected at 11 weeks of age, prior to the HFS diet challenge. Offspring sex affected *Lactobacillus* spp. C. cluster XI and *Roseburia* ($P \leq 0.001$; Table 5.4), where male offspring had significantly higher *Lactobacillus* spp., C. cluster XI and *Roseburia* than females ($P \leq 0.001$). Maternal treatment affected *Bacteroides,* C. cluster XI, and the relative abundance of Bacteroidetes ($P \leq 0.02$; Table 5.4). The HFS-CON offspring had fewer *Bacteroides* than Lean-CON and Control ($P \leq 0.03$) and had a lower abundance of Bacteroidetes than all other groups ($P \leq 0.022$). C. cluster XI was higher in HFS-CON offspring compared to Lean-CON and Control ($P \leq 0.04$). Total bacteria, Bifidobacteria, *Methanobrevibacter, C. leptum,* and C. cluster I abundance was significantly affected by the interaction of maternal treatment and offspring sex ($P \leq 0.05$; Table 5.4). Male HFS-CON offspring had significantly greater abundance than females ($P = 0.001$). Male offspring from HFS-CON dams had more Bifidobacteria and *C. leptum* than pups from Control dams ($P \leq 0.01$) and more C. cluster I than all other maternal treatment groups ($P \leq 0.002$). Male offspring from HFS-CON dams also had more C. cluster I than females from HFS-CON dams ($P < 0.0001$). In female offspring, CR offspring had significantly more *Methanobrevibacter* than Lean-CON and HFS-CON ($P \leq 0.03$), while Control offspring only had more *Methanobrevibacter* than the HFS-CON offspring ($P = 0.04$).

Table 5.4. Gut Microbiota at 11 Weeks in Offspring from Lean-CON, Control, CR or HFS-CON Dams.

	Sex	Lean-CON	Control	CR	HFS-CON	Txt	Sex	Txt x Sex
Total bacteria	M	1528577±263569[ab]	1620403±244563[ab]	1439168±244125[ab]	2135845±221579[a]	0.76	0.53	0.006
	F	1622727±166655[ab]	1892998±90487[ab]	1682758±148535[ab]	1173845±127065[b]			
Bacteroides/	M	206348±51497[a]	234669±37116[a]	162538±35510[ab]	150595±23615[b]	0.005	0.95	0.47
Prevotella	F	221906±44477[a]	247341±20883[a]	201099±20975[ab]	89843±15569[b]			
Bifidobacteria	M	57089±19926[ab]	16040±5348[a]	87425±30248[ab]	129036±35844[b]	0.05	0.02	0.05
	F	38560±11334[a]	34837±13541[a]	46944±14487[a]	33786±8224[a]			
Methanobrevibacter	M	305±41.0[a]	308±57.3[a]	275±49.4[a]	383±48.2[a]	0.09	0.0001	0.02
	F	1134±123[ac]	1630±256[b]	1681±177[c]	1090±162[a]			
Enterobacteriaceae	M	1251±575.4	611±163.9	802±340.9	1134±303.4	0.43	0.14	0.46
	F	6510±4057	393±102.9	8482±6939	1041±393.3			
Lactobacillus	M	414608±97996	191678±42536	230522±62690	261417±68324	0.34	0.001	0.12
	F	113912±34672	149401±47424	148776±31236	100695±23810			

C. coccoides	M	1384961±270347	1381770±270850	1092318±163355	1206188±246439	0.14	0.33	0.81
	F	1695949±243629	1715540±264896	1138887±180812	1166514±232071			
C. leptum	M	355277±158887^{ab}	133726±49484^{a}	403136±141243^{ab}	729532±223544^{b}	0.31	0.74	0.01
	F	629188±97458^{a}	496297±117646^{a}	304812±55981^{a}	313397±71421^{a}			
C. cluster XI	M	1841±557^{a}	1515±242^{a}	2102±608^{ab}	3664±617^{b}	0.02	0.0001	0.06
	F	456±74.1^{a}	608±80.7^{a}	516±57.7^{ab}	696±168^{b}			
C. cluster I	M	11575±2614^{a}	8630±1789^{a}	5602±1899^{a}	25129±5091^{b}	0.01	0.31	0.001
	F	10269±1764^{a}	13325±2196^{a}	11311±1233^{a}	8520±1728^{a}			
Roseburia	M	64186±12315	25769±5819	75538±29464	55271±22187	0.44	0.0001	0.46
	F	323±133.2	188±47.37	690±210.8	263±118.4			
Percent Bacteroidetes	M	13.1±2.4^{ab}	15.2±2.1^{a}	11.5±2.1^{ab}	7.0±0.7^{b}	0.0001	0.87	0.81
	F	12.9±1.6^{ab}	13.2±1.1^{a}	12.5±1.5^{ab}	7.5±0.6^{b}			

Values are mean ± SEM (n=8-12 per group). ^{a,b}Values without a common superscript are significantly different within a sex ($P<0.05$).

Following the HFS diet challenge, C. cluster I was affected by offspring sex (P=0.003; Table 5.5), where males had a greater abundance than females. Maternal treatment affected *C. leptum,* C. cluster XI and the percent of Bacteroidetes (P≤0.042; Table 5.5). Offspring from HFS-CON dams had significantly more *C. leptum* than the offspring from Control dams (P=0.036). The abundance of Bacteroidetes was higher in the CR group compared to the Lean-CON group (p=0.013). C. cluster XI was affected by maternal treatment (P=0.039) and there was a trend for Lean-CON offspring to have more C. cluster XI than all other groups, but it was not statistically significant.

The interaction of time and sex affected Bifidobacteria, *Roseburia* and *Methanobrevibacter* (P≤0.037). Both male and female offspring had a decreased Bifidobacteria in response to the 6 week HFS diet challenge (P≤0.007). With time the amount of *Roseburia* in male offspring decreased significantly (P<0.0001). *Methanobrevibacter* was affected by the interaction of time and sex (P<0.0001) as well as time and maternal treatment (P=0.034) wherein it increased in male offspring (P<0.0001), decreased in female offspring (P<0.0001) and increased in both the Lean-CON and the HFS-CON (P≤0.011) during the 6 week HFS diet challenge. C. cluster XI and the percent of Bacteroidetes were also affected by the interaction of time and maternal treatment (P≤0.032). C. cluster XI increased in offspring from Lean-CON dams from 11 to 17 weeks (P<0.0001), while the percent of Bacteroidetes increased in CR and HFS-CON offspring (P≤0.033). The interaction of time, sex and treatment affected *Lactobacillus* spp. and C. cluster I (P≤0.045). Male offspring in all treatment groups showed a decrease in the abundance of *Lactobacillus* spp. with time (P≤0.027) and female offspring in the CR group also decreased (P=0.042). Within the maternal treatment groups, C. cluster I had varying affects with male Lean-CON offspring increasing with time (P=0.005) and male HFS-CON offspring decreasing with time (P=0.014).

Table 5.5. Gut Microbiota at Sacrifice at 17 Weeks of Age in Offspring from Lean-CON, Control, CR or HFS-CON Dams.

	Sex	Lean-CON	Control	CR	HFS-CON	Txt	Sex	Txt x Sex
Total bacteria	M	1223343±36700	1787771±344180	1691735±151527	1735720±92432	0.32	0.24	0.48
	F	1668852±79702	1770551±213712	3972506±145349	3972506±2137970			
Bacteroides/	M	88449±8950	120514±18711	211750±25388	217888±41918	0.29	0.13	0.76
Prevotella	F	202893±19138	197086±25291	300308±45606	558372±357687			
Bifidobacteria	M	9012±2673	5249±2034	9140±2876	4620±3222	0.24	1.0	0.91
	F	9552±3751	7528±2360	7577±3105	3372±1340			
Methanobrevibacter	M	981±133	952±130	1024±79.3	1222±113	0.41	0.84	0.71
	F	1119±181	934±94.4	974±97.4	1081±119			
Enterobacteriaceae	M	756±193	2352±699	539±101	4009±3012	0.77	0.95	0.34
	F	4335±3911	1282±571	1299±389	1107±455			
Lactobacillus	M	29007±9116	46027±18233	8209±30506	80211±47623	0.93	0.48	0.24

	F	56272±34220	66525±25140	27919±10335	34664±10117			
C. coccoides	M	924056±96356	1683324±325054	1479280±170563	1796379±306810	0.31	0.33	0.44
	F	1280902±151762	1558111±322738	1421070±259006	8267835±6300707			
C. leptum	M	535812±113283^{ab}	526511±120460^{a}	799080±151672^{ab}	897344±209844^{b}	0.04	0.20	0.11
	F	1102637±192897^{ab}	557425±140724^{a}	534553±68526^{ab}	1242820±307728^{b}			
C. cluster XI	M	28373±13503	4426±1423	6193±2817	5995±1363	0.04	0.21	0.24
	F	9218±3840	5227±2237	5689±2053	4599±1706			
C. cluster I	M	29359±15813	7502±2182	8298±776	7702±1493	0.14	0.003	0.14
	F	905±432	986±270	876±482	1065±653			
Roseburia	M	932±279	561±297	622±221	407±285	0.67	0.07	0.47
	F	294±116	511±207	293±73.6	329±108			
Percent Bacteroidetes	M	7.4±0.9^{a}	10.8±2.4^{ab}	13.3±1.9^{b}	12.9±2.7^{ab}	0.02	0.07	0.25
	F	12.3±1.1^{a}	13.0±1.6^{ab}	17.5±2.3^{b}	11.0±1.3^{ab}			

Values are mean ± SEM (n=8-12 per group). ^{a,b}Values without a common superscript are significantly different within a sex ($P<0.05$).

5.4 Discussion

Our results did not support the hypothesis that pre-pregnancy maternal caloric restriction would result in improvements in offspring metabolic health. Specifically, body composition, glycemia and gut satiety hormone levels were not improved in offspring of dams who had weight loss induced via caloric restriction just prior to pregnancy. Interestingly, while maternal CR resulted in significant weight loss during the treatment phase, an overcompensation in weight gain appeared to occur during pregnancy and was comparable to gestational weight gain of the obese HFS fed rats (HFS-CON). Although there was no difference in body weight among offspring from the various maternal groups, offspring from the never obese Lean-CON dams did have the lowest percent body fat following the HFS diet challenge, which was significantly different from CR offspring. Furthermore, male offspring from CR dams had increased expression of SCFA receptors and along with male HFS-CON offspring had a decrease in hepatic expression of AMPKα1. Our investigation of offspring gut microbiota in response to maternal CR versus Control diet revealed differential sex effects.

Following breeding, all dams except the HFS-CON group were switched to the control diet *ad libitum* to ensure adequate gestational nutrition. Despite greater weight loss prior to pregnancy, the CR dams rapidly regained weight throughout gestation. During the pre-pregnancy treatment period, CR dams lost approximately 8 g which is in contrast to the approximate 18 g weight gain seen in the Lean-CON and Control groups and 35 g gain in the HFS-CON group. This weight rebound is contrary to the inverse relationship shown between the amount of weight loss and the amount of weight regain in adults who consumed a low calorie diet for 8 weeks (32). Subjects who experienced larger reductions in BMI during the 2 month long energy restriction phase had lower weight regain during the 6-month follow-up period (32). It is possible that the relationship observed in humans was not seen in the current study given that the metabolic demands and hormonal environment of pregnancy that immediately followed our weight loss phase represents a very unique state compared to that of free-living men and women post weight-loss.

Contrary to expected neither calorically restricting the obese dams pre-pregnancy or switching them from a HFS diet to a control diet, resulted in a decrease in offspring fat mass. Hull et al. (218) and Chandler-Laney et al. (219) showed that maternal obesity and excessive gestational weight gain are associated with increased fat mass in infants 1-2 days after birth and at 1 year following birth in humans. Given the persistence of obesity, it stands to reason that the higher fat mass of offspring exposed to maternal obesity and excessive gestational weight gain would be maintained or expanded following a HFS diet challenge. The findings of the present study in fact support this. The obese dams fed the control diet did not lose weight during the treatment phase and therefore entered pregnancy in the obese state; the CR dams did lose weight, but gained a large amount of weight during gestation. Together, the persistent maternal obesity on one hand and the excessive gestational weight gain on the other resulted in both groups having offspring with body fat percentages that were equal to the HFS-CON offspring in adulthood. However, a study by Hure et al. (220) failed to show an increase in body fat in the fetus of women with greater gestational weight gain which opens the possibility that the CR and Control offspring in our study had increased body fat as a result of the postnatal HFS diet challenge alone. Unfortunately, we are limited in not knowing the body composition of offspring just prior to the HFS diet challenge due to restrictions on animal movement between campus facilities. Future research in this area should determine if greater weight loss is required prior to conception and /or devise strategies in rodent models to control for gestational weight gain.

AMPK is a key regulator in glucose homeostasis throughout the body (221). In the liver and in skeletal muscle, AMPK plays a role in glucose production/uptake and lipid metabolism (221, 222). Specifically, AMPK activation decreases expression of key genes needed to generate lipids (222). AMPK is comprised of α, β and γ subunits (221, 222). There are 2 genes that code for the α subunit: AMPKα1 and AMPKα2 (221). Only AMPKα1 was measured in this study. CR has been shown to increase AMPKα2 expression but not AMPKα1 expression in skeletal muscle of mice (223). Although shown in the liver not in skeletal muscle, our results show a downregulation of AMPKα1 expression in offspring of CR dams compared to Lean-CON and Control dams. AMPK activation, specifically the α1

subunit, is very sensitive to the energy content of cells, which is regulated by the nutritional environment of the organism (224). Assifi et al. (224) investigated how hepatic AMPK responded to 48 hours of starvation followed by 24 hours of refeeding. After 24 hours of refeeding AMPKα1 activity was decreased by approximately 60% (224). A similar decrease in AMPK activation is seen in the skeletal muscle of mice in response to consumption of a high fat diet (225). Data from our HFS-CON offspring shows that hepatic AMPKα1 is also decreased compared to Control offspring. However, it is unclear whether the decrease in AMPKα1 mRNA levels in our offspring from CR and HFS-CON dams is a result of our 6 week HFS challenge in adulthood or if it is a result of fetal 'programming' effects. In any case, it appears that Lean-CON and Control maternal treatments may have protective effects on AMPKα1 mRNA levels in their offspring exposed to a HFS diet challenge. It is important to note that our data is limited to mRNA levels and as such we do not know about the activation of AMPK which could be further probed with Western blots. The sensitivity of AMPKα1 to the changes in the nutritional environment and the key role AMPK plays in whole body cellular metabolism potentially make it a key target for interventions trying to break the transgenerational cycle of obesity.

Gut microbiota also play an important role in the metabolism of their host and have been shown to affect AMPK expression in the liver (26, 211). At 11 weeks offspring from dams in the HFS-CON group had a microbiota profile reflective of obesity and consumption of a high fat diet, including more Firmicutes and less Bacteroidetes (211, 212). Offspring sex appeared to play a significant role in the abundance of bacteria within the HFS-CON offspring at 11 weeks. Sex specific differences in the gut microbiota were also shown by Markle et al. (226) wherein sex differences in gut microbiota appeared around puberty and persisted into adulthood. At 11 weeks male offspring from HFS-CON dams had more total bacteria than female offspring. This difference in total bacteria may have protective effects on female offspring. As previously discussed, a high fat diet is associated with a decrease in AMPK (225). Furthermore, diet appears to interact in an important way with the gut microbiota because germ free mice exposed to a high fat diet do not show the characteristic decrease in hepatic AMPK activation seen in conventionally raised mice fed a high fat diet

(162). Although the germ-free model is very unique, it is possible that lower microbial numbers in our female offspring could be associated with reduced AMPK suppression and better fat utilization than male rats with more total bacteria. In fact, studies looking at gene expression indicate that when exposed to a high fat diet female rats demonstrate higher fatty acid oxidation in skeletal muscle than male rats (227, 228) although neither study investigated the relationship between sex differences in gut microbiota and AMPK in high fat feeding.

Consumption of a high fat diet results in greater diversity in the microbial community of the gut via a decrease in Bacteroidetes and an increase in Firmicutes and other phyla (16). Our results correspond with this finding, wherein an increase in diversity in all offspring minimized the differences between maternal treatment groups. Our results did not show a typical increase in Firmicutes following the HFS diet challenge especially in the male offspring. Both *Lactobacillus* spp. and *Roseburia* decreased in all male offspring following the HFS diet challenge. This decrease is consistent with our previous findings (Chapter 4; MC Hallam and RA Reimer, unpublished results), but is not consistent with the findings of Mozes et al. (229) and Mujico et al. (212) who both found an increase in *Lactobacillus* spp. when rats consumed a high fat diet. This inconsistency may have arisen from methodological differences. Mozes et al. (229) used a primer that amplified both the genus *Lactobacillus* and the genus *Enterococcus*, while we used a primer that only amplified the genus *Lactobacillus.* Mujico et al. (212) used a similar primer, but their diet was composed of 60% fat while our diet was only 40% fat.

Maternal pre-pregnancy CR did not have a large impact on offspring gut microbiota at 11 weeks before the HFS diet challenge with the exception of female offspring from CR having more *Methanobrevibacter* than the Lean-CON and the HFS-CON. Weight loss is associated with a decrease in gut microbiota stability, so it is possible that instability in microbiota of the CR dams precluded any further significant changes in their gut microbiota (230). Hoffmann et al. (231) showed that *Methanobrevibacter* is positively associated with long term and acute consumption of high sugar diets. Our results showed the same response in the abundance of *Methanobrevibacter* following the HFS diet challenge in offspring from

the Lean-CON, HFS-CON and all male offspring. Interestingly, female offspring showed the opposite and had a decrease in *Methanobrevibacter* following the HFS diet. This is another example illustrating the importance of investigating sex specific effects of diet and microbiota.

5.5 Limitations

The two major limitations of this study are the lack of fetal programming studies to compare our results to and that gestational weight gain was not controlled. Pre-pregnancy maternal obesity treatments are novel, which means there is a lack of strong scientific evidence to validate our results. A lack of evidence makes it difficult to determine which of our results show long term programming effects and which show the effects of the HFS diet challenge. Gestation is another key period of plasticity where the offspring are sensitive to the environment. If any beneficial programming effects resulted from pre-pregnancy CR, the relatively high amount of gestational weight gained may have removed them. Any future pre-pregnancy interventions need to incorporate strategies to control the amount of gestational weight gain.

5.6 Conclusion

Calorically restricting obese dams or switching to a control diet prior to pregnancy did not produce long lasting effects in offspring in our model. Given that developmental programming can occur *in utero* as well as during early postnatal life, future research on pre-pregnancy interventions should control for the independent effects of gestational weight gain and suckling environment. Only half of pregnancies are planned, so developing an intervention that is appropriate for pre-pregnancy, gestation and lactation will increase the impact on developmental programming because it will provide a more stable nutritional environment throughout the critical periods of plasticity. AMPK is a highly sensitive energy sensor that regulates metabolism throughout the body making in ideal target for fetal programming interventions. More research is needed to determine if the differences seen in AMPKα1 mRNA levels with CR in this study have any measurable physiological effect. Finally, striking differences were seen between male and female offspring microbiota that may have important implications in host metabolism potentially through the mechanism of

AMPK and may explain some of the sex specific effects of obesity programming identified in the literature.

Chapter Six: **General Discussion & Conclusion**

6.1 Introduction

Obesity in women of childbearing age is increasing worldwide and alongside it the associated co-morbidities including subfecundity and poor pregnancy outcomes. Furthermore, exposure to an adverse *in utero* environment leads to the perpetuation of obesity and metabolic disease or the transgenerational cycle of obesity. Improving maternal weight status prior to conception can have beneficial effects on obesity-related infertility, pregnancy outcomes and offspring health. Finding safe and non-invasive weight loss interventions that are readily accessible may be one way to interrupt the transgenerational cycle of obesity and subsequently improve the health of the global population. The three manuscript-based chapters summarizing this thesis work were designed to investigate the effects of four maternal pre-pregnancy treatments on maternal and offspring health. There were three main objectives of this thesis work including:

1) To determine the effects of a combined therapy of OFS+Sitagliptin prior to pregnancy on the maternal fecundity and pregnancy outcome of obese female Sprague-Dawley rats;
2) To determine the effects of the maternal OFS+Sitagliptin treatment prior to pregnancy on offspring metabolic health including body composition, glucose control, satiety hormones, gene expression and gut microbiota;
3) To determine the effect of a caloric restriction maternal treatment prior to pregnancy on offspring metabolic health including body composition, glucose control, satiety hormones, gene expression and gut microbiota.

6.2 Strengths & Limitations

6.2.1 Animals

Sprague-Dawley rats were chosen for this work because when exposed to a high fat/ sucrose diet they are a good model of common human obesity and because they have been used extensively as a model in programming literature (153, 215). When exposed to a "westernized" diet high in fat and sugar these rats develop two different phenotypes (232-234). Those phenotypes are diet-induced obese prone (DIO-P) and diet-induced obese resistant (DIO-R). This phenotype starts to emerge in rats after just 5 days of high fat

feeding (233). DIO rats are characterized by greater changes in body weight during the first week of high fat diets, increased caloric intake, decreased insulin sensitivity (233) and increased body fat percent (234). The proportion of rats that develop the DIO-P phenotype is usually around one half, but has been seen as high as two thirds in a given cohort of rats (232). In a study by Archer et al. (235) male Sprague-Dawley rats fed a high fat diet did not distribute into the typical DIO-P or the DIO-R phenotypes. The authors concluded that DIO-P characteristics may also be related to breeding colony (235). Although most of the research regarding the DIO-P phenotype has been in male rats there is also good evidence to suggest that female Sprague-Dawley rats also develop this phenotype (236, 237). The DIO protocol has been successfully established in our lab previously for both male and female Sprague-Dawley rats. In this study, 120 rats were purchased from Charles Rivers (Charles River, Quebec, Canada) and fed a HFS diet for 8 weeks before the top 50th percentile was selected to continue into the intervention portion of the study. To further enhance the DIO phenotype, the rats were fed the HFS diet for an additional 6 weeks for a total of 14 weeks.

6.2.2 Maternal Pre-pregnancy Treatments

One of the strengths of this work is that the maternal pre-pregnancy treatments are all applicable and readily transferable to human populations. Chapter 3 compares the maternal fecundity and the pregnancy outcomes for all 7 treatment groups. For the sake of clarity, the offspring from the different maternal treatment groups were divided between Chapters 4 and 5. Chapter 4 looked at the four main experimental groups which included the control, OFS, sitagliptin and the OFS+Sitagliptin groups while, Chapter 5 included the Lean-CON, Control, HFS-CON and the CR reference group.

6.2.2.1 Control Groups

A major strength of this study was the inclusion of numerous control groups. The Lean-CON dams were never obese and never consumed the HFS diet thereby providing a stable programming environment that could be considered normal and healthy (Table 6.1). By including this group it allowed us to determine if any of our maternal pre-pregnancy treatments could entirely reverse the programming effects of exposure to a HFS diet and result in offspring with a truly lean phenotype. The HFS–CON dams consumed the HFS diet

prior to conception to induce obesity and continued to consume the diet until they were euthanized when the pups were weaned (Table 6.1). Having a HFS-CON allowed us to not only to make conclusions about how protective treatments are compared to one another, but also how protective treatments compare to continual exposure to maternal obesity and excessive energy intake. Additionally, there was a control group (Control) where dams consumed the HFS diet during the obesity induction period but were subsequently switched onto the control (AIN-93) diet for breeding, pregnancy and lactation (Table 6.1). This control group allowed us to separate the effects of simply switching obese dams from a high fat to a control diet from the effects of our dietary and pharmacological treatment in obese dams. Finally, we include a group that was weight-matched to the combination treatment group (the CR group). While this group was intended solely to control for the independent effects of weight loss in the OFS+Sitagliptin group (versus weight loss and exposure to OFS and sitagliptin), it provided additional unique insight into the effects of maternal caloric restriction prior to pregnancy and thus was discussed in its own chapter.

Table 6.1 Diet Consumed by Dams and Offspring for Each Phase of the Experiment.

Phase	Lean-CON	Control	OFS	Sitagliptin	OFS+ Sitagliptin	Caloric Restriction	HFS-CON
Dams							
Obesity induction	AIN-93M	HFS	HFS	HFS	HFS	HFS	HFS
Maternal Treatment	AIN-93M	AIN-93M	AIN-93M + 10% OFS	AIN-93M + sitagliptin	AIN-93M + 10% OFS + sitagliptin	~30% less AIN-93M than OFS+ Sitagliptin	HFS
Breeding-Weaning	AIN-93G	AIN-93G	AIN-93G	AIN-93G	AIN-93G	AIN-93G	HFS
Offspring							

Weaning - 11 weeks	AIN-93G	AIN-93G	AIN-93G	AIN-93G	AIN-93G	AIN-93G	HFS
11 -17 weeks	HFS	HFS	HFS	HFS	HFS	HFS	HFS

6.2.2.2 OFS

Oligofructose was the prebiotic fibre selected as the maternal pre-pregnancy dietary treatment. OFS can be consumed as a dietary fibre (intact in plants) or as a functional fibre added to other foods because of the resultant health benefits (113). The high solubility of OFS allows it to be dissolved and easily incorporated into many different food products and beverages (113). The dose of OFS in human studies usually varies from approximately 10 to 20 g/day (109). One study in humans with an exceptionally high dose started with 15 g/day of OFS and then increased by 10g per week until a maximum of 55g/day was being consumed (125). Consumption of OFS is well tolerated with a few mild side effects that may include bloating and flatulence, which usually subside as the colon adapts (109). In animal models doses of dietary fibre as high as 30% (wt/wt) have been used without deleterious side effects (238). The dosage chosen for this study was 10% OFS (wt/wt), which means 10% of the weight of the diet provided to the rats was from OFS. Such a dose is commonly utilized in the literature (100, 108, 239) and could be tolerated by humans although doses in the magnitude of 5% are typically the maximum studied and provide similar metabolic benefits as seen in rodents (20). According to the Government of Canada, the daily recommended intake of fibre is 38 g/day for men and 25 g/day for women (240). The average daily intake of fibre, however, is only 16 g (240), which implies that the addition of OFS as a functional fibre to the human diet would likely result in health benefits for the majority of the population.

6.2.2.3 Sitagliptin

The brand name for sitagliptin is Januvia®. The prescribed dose of sitagliptin in humans is 100 mg per day for a patient with good renal clearance (143). That dose is reduced to 50 mg per day in people with moderate renal insufficiency and to 25 mg per day

in people with severe renal insufficiency or end stage renal disease (143). In this work the dams were given 10 mg/kg of sitagliptin daily. Although this dose is relatively high compared to the human dose, it is consistent with what is seen in other rodent models investigating sitagliptin and is in fact much lower than some recent studies in rats (31, 147). Sitagliptin has been used to treat T2D since 2006, but there is virtually no readily identifiable human literature regarding use of sitagliptin during pregnancy or its effects on human fecundity. Sitagliptin is in the pregnancy category B, indicating that animal studies have shown that it is not a risk to the developing fetus, but no well-controlled human trials have been conducted (142, 143). Additionally, it is unknown if sitagliptin would appear in human breast milk (142, 143). Metformin, another T2D drug, has been shown to be safe for use in women before and during pregnancy (241). With the increasing prevalence of T2D in women of child bearing age, it may be an increasing possibility that women could be taking sitagliptin prior to conception and during the early stages of gestation. Further investigation in humans should be done to determine if sitagliptin is safe for use in overweight and obese women of reproductive age; especially given that our results showed an increased prevalence in delivering a small litter in the sitagliptin groups (Table 3.3) and the fact that overweight and obese women are already at an increased risk of impaired fecundity.

Obesity is a complex disease, so it is no surprise that there is no easy solution. Obesity results from the combined interactions of many factors including environment, genetics, developmental programming, energy intake and activity levels (15, 17), so any effective treatment must address some if not all of these factors. Sitagliptin is already used in combination with other diabetes drugs and exercise interventions in treating T2D. Combining multiple interventions to enhance the effects of each individual treatment in an attempt to tackle obesity is intuitive and justified. This rationale was the basis for our examination of the combined treatment of OFS and sitagliptin.

6.2.2.4 Caloric Restriction Reference Group

The caloric restriction group was included for two reasons. First, it acted as a weight matched control for the OFS+Sitagliptin group. As mentioned, this allowed us to make conclusions about the combined treatment independent of any weight loss effects. To weight

match the OFS+Sitagliptin group, the dams were exposed to an approximate 25-30% caloric restriction. Finding treatments that result in unconscious weight loss with additional benefits beyond weight loss alone could revolutionize the way obesity is treated. The ability to show effects above a beyond weight loss may also be important in recruiting key stake holders to adopt a new treatment strategy for obese patients and ultimately improve population health. Secondly, we were able to investigate the fetal programming effects of caloric restriction, which is a common practice among women of childbearing age (73, 74). The potential risks or benefits of pre-pregnancy 'dieting' on offspring health is important, so that it can be addressed by health care providers counselling women who have the potential to become pregnant.

6.2.3 Experimental Design

One strength of this study was the duration of the obesity induction and treatment phases. The obesity induction period was a total of 14 weeks, which is more than sufficient to induce obesity in the Sprague-Dawley model (232, 236) while the treatment phase was 8 weeks long (Figure 1.1). Four weeks of 10% (wt/wt) OFS treatment is sufficient to produce changes in proglucagon expression, body weight and glucose control in rats and humans (108, 109). Similarly, at 3 mg/kg, a dose much lower than the dose in our study, sitagliptin lowers blood glucose levels, plasma insulin and decreases triglycerides and cholesterol after only 4 weeks of treatment in Sprague-Dawley rats (140). Furthermore, other rat fetal programming studies that involve maternal pre-pregnancy interventions have used interventions ranging from 1 week to 6 weeks before breeding (96, 112, 188). The intervention time of 8 weeks, twice the time it takes to see effects of OFS and sitagliptin, was chosen to ensure that the treatments would bring about enough change in the dams to cause any potential positive effects on offspring health.

This study may have been strengthened if the maternal pre-pregnancy intervention was determined by a specific weight loss goal as opposed to an 8 week time period. One human study investigating the effects of weight loss on obesity-related infertility following bariatric surgery showed that a reduction of body weight of approximately > 5 kg/m^2 or a final BMI of 34 kg/m^2 was required for improvements in fertility and pregnancy outcomes

(85). The ongoing LIFESTYLE study in the Netherlands aims to reduce the need for fertility treatment in overweight and obese women by reducing their body weight 5-10% (92). In the present work only the OFS+Sitagliptin and the CR dams lost weight in the pre-pregnancy intervention and they only experienced an average weight reduction of 2.5%. The weight reduction of 2.5% did not bring the dams anywhere near to 200-225 g, which is the optimal weight for reproduction in female rats (40). Had our intervention been based on a specific weight reduction goal that resulted in dams achieving a body weight closer to the ideal breeding weight, it might have resulted in improved maternal fecundity, pregnancy outcomes and offspring health.

The mismatch hypothesis suggests that a mismatch between the pre-natal environment and the childhood and adulthood environment results in metabolic disease (242). The fetus is programmed for the future environment base on *in utero* cues and cues from the periconceptual phase (242). Disease results when the programmed phenotype does not match the environment (242). Alterations in the maternal nutritional environment especially around the time of conception and throughout gestation may result in a greater mismatch due to a lack of consistent cues. With the exception of dams in the Lean-CON, HFS-CON and the Control group, all of the dams were switched onto *ad libitum* control diet (AIN-93) for pregnancy and lactation (Table 6.1). The mismatch between the pre-pregnancy intervention and *ad libitum* control diet may have independently blunted any offspring programming effects if in fact there were any (Table 6.2).

Table 6.2. The Macronutrient Mismatch between Intervention Diets

% of Total Calories	Control (AIN-93)	OFS	HFS
Carbohydrates	63.8	65.3	49.8
Protein	19.5	18.6	11.1
Fat	16.7	16.1	39.1

When the offspring reached early adulthood they were exposed to a 6 week HFS diet to determine if the maternal pre-pregnancy treatments would affect their ability to adapt to an environment of overconsumption (Figure 1.1). Sprague-Dawley rats reach sexual maturity at 10 weeks of age (243), so the high fat diet was implemented at 11 weeks to coincide with young adulthood. Exposure to a high fat diet for 6 weeks is long enough for rats to present with an obese phenotype of increased body weight, adiposity and early indicators of insulin resistance (233). Previous fetal programming studies have shown the combined effects of maternal diet and offspring diet, specifically that when pups of high fat fed dams consume a high fat diet postnatally the negative metabolic effects are exasperated in the offspring (244). Given the current obesogenic environment, it is important to determine whether or not programming effects resulting from maternal pre-pregnancy treatment help or hinder the ability of offspring to adapt when exposed to a high fat diet. The ability to investigate whether a maternal pre-pregnancy treatment results in programming effects and that those programming effects are permanent enough to endure a HFS diet challenge is a major strength of this experimental design.

6.2.4 Gene Expression via Real-Time Polymerase Chain Reaction (PCR)

Real time PCR was used to determine GPR41 and GPR43 mRNA expression in the proximal colon and hepatic AMPKα1 mRNA expression. Real time PCR is a simple technique that is highly sensitive and specific and for that reason it is considered the current gold standard for measuring gene expression (245). Despite the strengths of real time PCR as a technique, it is difficult to make meaningful conclusions about offspring metabolic health based on gene expression data alone because we do not know if the mRNA is being used to create a functional end product. For instance, measuring mRNA expression of AMPKα1 does not indicate if AMPK is activated or not, which makes it difficult to make any reasonable conclusions based solely on gene expression. Further investigation of the downstream products of GPR41, GPR43 and AMPKα1 through Western blots could have provided further insight into the differences in offspring metabolism resulting from maternal pre-pregnancy treatment.

6.3 Overall Summary & Interpretation of Results

Overall the findings of this thesis work did not support the proposed hypotheses. The hypothesis of aim 1 was that the combined OFS+Sitagliptin treatment would produce the greatest improvements in maternal fecundity and pregnancy outcomes for obese female Sprague-Dawley rats. Our aim 2 hypothesis was that maternal OFS+Sitagliptin treatment would result in the greatest improvements in glucose control, satiety hormone levels and body composition in their offspring. Finally, in Chapter 5, we investigated the hypothesis that offspring born to CR dams would have improved glucose control, satiety hormone response and body composition. Additionally, the microbiota of offspring was also analyzed to determine which maternal treatments resulted in the healthiest gut microbiota profile. Chapter 3 investigated the effects of maternal pre-pregnancy treatment on maternal fecundity and pregnancy outcomes and those findings have been summarized in Table 6.3. The offspring outcomes were divided into two chapters and analysed separately and those findings have been summarized in Table 6.4. Additionally, a detailed summary of the gut microbiota findings can be found in Table 6.5. Tables and figures showing the comparison between all seven maternal pre-pregnancy treatment groups can be found in Appendix C. Our main findings from Chapters 3-5 are: 1) pre-pregnancy maternal obesity treatment did not have beneficial effects on maternal pregnancy outcomes and did not result in protective programming for offspring; 2) pre-pregnancy maternal obesity treatment is associated with changes in SCFA receptor and hepatic AMPK mRNA expression (Figure 4.3 & Figure 5.4); and 3) pre-pregnancy maternal OFS treatment has protective effects on offspring microbiota and showed a trend towards other offspring health benefits (Table 4.4 & Table 4.5). This final summary will discuss the results from Chapters 3-5 in the context of our three main findings.

6.3.1 Pre-pregnancy Maternal Obesity Treatment Did Not have Beneficial Effects on Maternal Pregnancy Outcomes & Did Not Result in Protective Programming for Offspring

A pre-pregnancy treatment with CR resulted in significant weight loss during the treatment phase (Table 3.1). During pregnancy, however, CR dams gained the most weight which was equivalent to the HFS-CON dams (Table 3.1). It is not surprising that after the

CR dams were provided with *ad libitum* access to control diet during pregnancy they gained excessive weight, especially given the literature-supported association between weight loss via caloric restriction and weight regain (32). The CR dams continued to gain weight during lactation and gained more weight than any other group during that period (Table 3.1). Although there are no specific gestational weight gain recommendations for rodent models, one study found that normal weight rats fed a standard laboratory chow diet *ad libitum* gained approximately 42 g during gestation (246). In our study the female rats were also fed a control diet *ad libitum* throughout gestation and lactation, although it was a defined AIN-93 diet versus chow. Dams in the other treatment groups did not gain as much weight as CR and HFS-CON during gestation, but all dams gained more than 42 g; therefore, excessive maternal weight change during pregnancy and lactation may have in fact blunted any beneficial offspring outcomes from maternal pre-pregnancy treatment. Furthermore, in humans, excessive gestational weight gain is associated with increased risk of gestational hypertension, preeclampsia, cesarean-section delivery, spontaneous abortion and small or large for gestational age infants (247). Children born to mothers who gained above the Institute of Medicine (IOM) guidelines for gestational weight gain during pregnancy have increased risk for obesity and increased central adiposity (248). The 2009 Institute of Medicine (IOM) guidelines recommend different amounts of gestational weight gain according to maternal BMI. For underweight women it is recommended that they gain a total of 12.5-18 kg (28-40 lbs); normal weight women should gain 11.5-16 kg (25-35 lbs); overweight women should gain 7-11.5 kg (15-25 lbs) and obese women should gain 5-9 kg (11-20 lbs) (249). Not only is it possible that excessive gestational weight gain prevented beneficial programming from occurring, but it is also possible that high gestational weight gain contributed to negative programming in offspring. More research is needed to determine a healthy range of gestational weight gain in overweight and obese rats.

The OFS+Sitagliptin dams had significant weight loss during the treatment phase and gained the least amount of weight during pregnancy (Table 3.1). Despite weight loss prior to pregnancy and low gestational weight gain, the female pups from OFS+Sitagliptin dams were significantly heavier at birth (Table 3.1). The criteria for pups to be considered

macrosomic is that they weigh greater than 1.7 standard deviations above a lean control mean (250) and the female OFS+Sitagliptin pups were below the cut off of 8.7 g. Although the female OFS+Sitagliptin pups were significantly heavier they did not meet the criteria for large for gestational age, so this should not have negated any potential programming effect of the maternal pre-pregnancy treatment in these offspring.

As discussed in Chapter 3, significantly more dams from the sitagliptin treatment group had small litters which was defined as <10 pups (Table 3.3). OFS+Sitagliptin rats also tended to have small litters although it was not statistically significant (Table 3.3). At birth the litters were culled to 10 pups with 5 males and 5 females where possible to control for the effects of litter size on obesity programming. Pups from small litters are exposed to overfeeding, which is associated with higher body and fat weight, higher plasma insulin levels and negative alterations in gene expression (251). Rearing practices and maternal age are also associated with litter size. Female rats reared with both male and female siblings give birth to larger litters (40). The conditions in which rats were reared are unknown because the female rats were received at 10 weeks of age from Charles River. Female rats give birth to the largest litters and highest birth rates when they are 70-105 days old. At the start of breeding all of the dams were roughly 210 days old, so this may have affected the prevalence of small litter and adverse outcomes; however, any effects would have been consistent across all maternal treatment groups. Exposure to a smaller litter size may have predisposed offspring from the sitagliptin and the OFS+Sitagliptin group to increased risk of obesity or have negated any effects of the maternal pre-pregnancy treatment. Research studies investigating the effects of small litter size on offspring metabolism compare litters of 3 pups to litters of 10 or more pups (251, 252). For the sitagliptin dams, litter size ranged from 5-10 pups with an average litter size of 8 pups (Table 3.2), so it is difficult to make any firm conclusions about the effect of litter size on sitagliptin pup outcomes. Litter size was not included in the statistical analysis because not all of the maternal treatment groups are adequately represented in the each litter size category. It would be important for future work related to this study to use cross-fostering of pups to ensure equal litter size across all treatments.

When the relationship between pregnancy outcome and pup weight at birth and weaning was examined, we found that adverse pregnancy outcomes were associated with lower body weight in both male and female pups (Table 3.4). The definition of adverse pregnancy outcome was maternal death or ≥ half of pups deceased within 1 week. Low birth weight is associated with increased risk for metabolic disease, heart disease and stroke (59). Incidence of low birth weight can result from IUGR (253) and IUGR is associated with offspring programming resulting in increased adiposity in adulthood (254). The EPOCH study, a retrospective cohort looking at children exposed to IUGR, also found that growth restriction was associated with increased abdominal fat and insulin resistance in 10 year old children (255). Given the relationship between low birth weight, IUGR and adipose tissue accumulation we can speculate that any potential improvements in offspring percent body fat as a result of maternal pre-pregnancy treatment may have been shrouded by increased body fat in pups from dams that experienced adverse pregnancy outcomes.

Table 6.3. Overall Summary of Findings for Maternal Fecundity and Pregnancy Outcome.

	Lean-CON	**Intervention Groups**	**HFS-CON**
Treatment Weight Δ	Normal weight gain	Weight loss in OFS+S and CR	Highest weight gain
Pregnancy Weight Δ	↑ weight gain vs. C,OFS+S	↑ weight gain in S vs. C,OFS+S	↑ weight gain vs. C,OFS, OFS+S
Lactation Weight Δ	≈	Weight loss in OFS and S; Greatest weight gain in CR	≈
Pregnancy Outcome	≈	↑ small litter in S vs. C	≈
Birth Weight	≈	↑ birth weight in OFS+S vs. C females	≈
	Adverse pregnancy outcome was associated with low birth and weaning weight independent of maternal pre-pregnancy treatment group		

6.3.2 Pre-pregnancy Maternal Obesity Treatment is Associated with Changes in SCFA Receptors & Hepatic AMPK mRNA Expression

The combined treatment of OFS+Sitagliptin had the highest proximal colon GPR 41 and GPR 43 mRNA levels (Figure 4.3) and in Chapter 5, CR was associated with a significant increase in proximal colon GPR41 and GPR43 expression (Figure 5.4). In Chapter 4 we speculated that the increase in GPR41 and GPR43 was a side effect of increased Gα proteins intended for increased production of GLP-1 receptors. It is possible that the effects of CR on SCFA receptor gene expression may also be related to Gα proteins except with a different trigger. A study by Hadjimarkou et al. (256) used an antisense oligodeoxynucleotide probe to inhibit certain Gα subunits in rats both during and after exposure to a 24 hour period of fasting. They found that inhibition of certain Gα subunits resulted in greater weight loss during fasting and less weight regain than controls (256). Since weight and appetite reduction occurred when Gα subunits were inhibited it can be inferred that specific Gα subunits may play a role in body weight regulation and may protect against weight loss. In the present study, dams in the CR group were also exposed to a period of feed restriction followed by a return to *ad libitum* feeding. Offspring whose mothers were exposed to weight loss via CR immediately prior to conception may have been programmed to have an increased amount of the specific Gα proteins that will protect them from weight loss. Increased GPR41 and GPR43 expression in offspring from OFS+Sitagliptin dams could also be a programming effect intended to protect against weight loss, because both the CR and the OFS+Sitagliptin dams lost weight during the treatment phase. Further research should measure GLP-1 receptors and investigate the specific Gα protein subunits to help determine the cause of increased GPR41 and GPR43 expression and if it is beneficial or detrimental of offspring health.

Sitagliptin and OFS+Sitagliptin had significantly lower AMPKα1 mRNA levels than the Control and OFS groups (Figure 4.3) in Chapter 4 while both CR and HFS-CON were associated with the lower AMPKα1 expression (Figure 5.4) in Chapter 5. Sitagliptin has no effect on phosphorylated AMPK or AMPKα2 expression in cardiac muscle of mice (257), so it is unlikely the maternal pre-pregnancy sitagliptin treatment programs offspring to have

lower AMPKα1 expression. In Chapter 5, we discussed how it was difficult to determine whether the decrease in AMPKα1 is a result of the pre-pregnancy maternal treatment or if it is the result of the HFS diet challenge given to offspring, since consumption of a high fat diet is associated with decreased AMPK activation (225). Given that sitagliptin is not associated with a reduction in AMPK expression it can be concluded that, hepatic AMPKα1 expression in offspring from mothers that were in the Lean-CON, Control and OFS treatment groups may be protected against a high fat diet.

Table 6.4. Overall Summary of Findings for Offspring Programming Effects at 17 weeks of Age.

Chapter 4	Chapter 5
	Body Composition:
	↓ % body fat, fat mass (Lean-CON vs. CR)
Plasma (fasting):	Plasma (fasting):
↓ blood glucose (S vs. C)	↑ leptin (Lean-CON vs. C)
↑ leptin (OFS, OFS+S vs. C)	↑ ghrelin, GIP (Lean-CON vs. HFS-CON)
↓ ghrelin (OFS vs. OFS+S)	
GI characteristics:	GI characteristics:
↑ stomach weight(S vs. OFS,C)	↓ stomach weight (Lean-CON vs. CR)
↓ sm. intestine length, weight (OFS vs. S, C)	↓ sm. intestine weight (Lean-CON vs. C)
mRNA expression:	mRNA expression:
↑ GPR41, GPR43 (OFS+S)	↑ GPR41, GPR43 (CR males)
↓ AMPKα1 (S, OFS males)	↓ AMPKα1 (CR, HFS-CON males)

6.3.3 Pre-pregnancy Maternal OFS Treatment has Protective Effects on Offspring Microbiota & Showed a Trend towards Other Offspring Health Benefits

The gut microbiota of all offspring was analyzed at 11 weeks prior to the HFS diet challenge and at 17 weeks immediately following the high fat diet challenge. Maternal pre-pregnancy OFS treatment had protective effects on Bifidobacteria, because the amount of Bifidobacteria in offspring from OFS dams stayed the same between 11 and 17 weeks (Table

4.4 & 4.5). All other maternal treatment groups responded to the HFS diet challenge with the typical increase in the abundance of Firmicutes, decrease in the abundance of Bacteroidetes and decrease in Bifidobacteria. Despite these protective effects, there was no significant correlation between maternal Bifidobacteria abundance and offspring weaning Bifidobacteria*;* the only weaning gut bacteria to be correlated with maternal feces at birth were in the Clostridia class of the Firmicutes phyla (Figure 3.1). Currently, there is little literature investigating pre-pregnancy treatment effects on maternal feces at birth and the subsequent effects on the next generation at weaning. However, there is one study that looked at the succession of Bifidobacteria in humans between mother and infant and found that the highest dissimilarity between maternal and offspring Bifidobacteria occurred between late pregnancy and early infancy and that similarity increased as the child got older (183). Bifidobacteria is associated with several metabolic benefits including a negative correlation with body weight and visceral fat mass (24), which coincides with our finding that OFS tended to have lower percent body fat. Offspring from both the OFS and OFS+Sitagliptin maternal pre-pregnancy treatment had higher leptin than Control offspring (Table 4.2) while ghrelin was lower in OFS compared to OFS+Sitagliptin (Table 4.2). These findings suggest that offspring whose mothers consumed OFS have better fasting appetite regulation, which may also have contributed to their tendency to have lower fat mass. As discussed above, OFS may also have protective effects on AMPKα1 expression when exposed to a high energy diet. This may also explain the lower body weight displayed in offspring of OFS treated dams, because AMPK decreases body fat accumulation. Despite only small effects on offspring health, OFS may still have potential to have protective programming effects due to significant changes in offspring appetite regulation and gut microbiota in this experiment.

Table 6.5. Overall Summary of Findings for Microbiota.

	Firmicutes	Bacteroidetes & Actinobacteria	Proteobacteria & Archea
Birth*	↑ *Lacto* (Lean-CON vs. HFS-CON, NL**) ↑ C. clust XI (A vs. NL) ↑ *Rose* (C vs. Lean-CON, OFS, OFS+S, CR, HFS-CON)		
Weaning	↑ C. clust I (CR females)	↑ *Bacter/Prev* (A vs. SL males)	↑ *Entero* (NL vs. SL males; C females) ↑ *Methano* (HFS-CON vs. Lean-CON, C,CR males)
11 weeks	↑ *Lacto* (S vs. OFS+S) ↑ *Rose* (OFS+S males) ↑ C. clust XI (HFS-CON vs. Lean-CON, C) ↑ C. *lept* (HFS-CON vs. C males) ↑ C. clust I (HFS-CON males)	↑ *Bacter/Prev* (S vs. OFS+S) ↑ Bifido (OFS+S vs. OFS) ↑ *Bacter/Prev* (Lean-CON, C vs. HFS-CON) ↑ Bifido (HFS-CON vs. C males)	↑ *Entero* (OFS+S vs. C) ↑ *Methano* (CR vs. Lean-CON, HFS-CON; C vs. HFS-CON females)
17 weeks	↑ *Rose*, *Lacto* (C vs. S) ↑ C. *lept* (HFS-CON vs. C)	↑ *Bacter/Prev* (CR vs. Lean-CON)	↑ *Entero* (C vs. OFS, S)

*Maternal feces at birth

** normal litter, NL; small litter, SL; adverse, A

Lacto, Lactobacillus; Rose, Roseburia; Bacter/Prev, Bacteroides/Prevotella; Entero, Enterobacteriaceae; Methano, Methaonbrevibacter; C. *lept*, C. *leptum*; Bifido, Bifidobacteria

6.4 Future Directions

Despite a lack of significant findings, this thesis work is a valuable starting place for other fetal programming studies that incorporate maternal interventions. First, future experiments should incorporate measures to control weight gain in gestation and lactation to ensure the programming cues given to the growing fetus/infant are consistent. In order to appropriately control weight gain during pregnancy and lactation specific guidelines about weight gain/ loss should be determined for the rodent model. Secondly, any interventions that use a pharmacological agent need to ensure that the agent is safe to use during and around pregnancy. This is extremely important and practical because many pregnancies are not planned, so implementing any obesity treatment in women of child bearing age should be safe for use in early pregnancy.

Given the protective effect maternal pre-pregnancy OFS treatment had on Bifidobacteria during the HFS diet challenge and the trend for decreased percent body fat, further investigation into the programming effects of OFS is warranted. Since, OFS is naturally found in food products it may be safe to be consumed during pregnancy and lactation. Studies investigating the dosage and safety of gestational OFS consumption are needed. If proven safe, OFS treatment throughout the periconceptual phase until lactation might provide a stable enough maternal nutritional environment to produce significant beneficial programming effects.

Both hepatic AMPKα1 and proximal colon GPR41 and GPR43 expression were affected by maternal treatment. SCFA receptors GPR 41 and GPR 43 were significantly affected by maternal pre-pregnancy OFS+Sitagliptin and CR treatment. For both treatments, the suggested mechanisms include Gα proteins, so future work should measure the abundance of specific Gα protein subunits and GLP-1 receptors in the colon. Quantifying SCFA would also provide valuable insight into how the pre-pregnancy maternal treatments are affecting SCFA receptor gene expression. AMPKα1 gene expression was also affected by pre-pregnancy treatment. Additional tests investigating genes upstream from AMPKα1 may help to elucidate the causal mechanism involved. For both hepatic AMPKα1 mRNA levels and proximal colon GPR41 and GPR43 mRNA expression, further work should use

the stored tissues collected during this experiment to determine if there are any downstream gene effects through Western blots and protein assays.

As indicated in Chapter 5, more research is necessary to determine gender specific characteristics on microbiota. Offspring sex affected how all groups responded to the HFS diet challenge and sometimes male and female offspring had responses that were completely opposite to each other. Further research should compare the differences between male and female gut microbiota in regards to high fat feeding, OFS consumption, sitagliptin treatment and caloric restriction. Understanding the sex specific responses to all of the maternal treatments would allow us to make stronger conclusions about the programming effects of maternal pre-pregnancy treatment on offspring gut microbiota.

6.5 Conclusions & Significance

Overall it appears that despite significant effects on gene expression in the proximal colon and liver none of the pre-pregnancy maternal treatments investigated in this work improve maternal fecundity, pregnancy outcome or the metabolic health of offspring. Our results indicate that maternal treatment with OFS may still have potential to improve offspring health. We have also uncovered several ways to improve future experiments investigating fetal programming through maternal interventions. A large number of people worldwide are overweight and obese and the transgenerational cycle of obesity continues to rage on through the present generation. It is important for researchers to continue seeking out novel and effective ways to reduce the burden of obesity and its related co-morbidities.

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APPENDIX A: DIET COMPOSITION

Table A.1 Composition of Experimental Diets.

g/kg	AIN-93G	AIN-93M	OFS	HFS
Cornstarch	397	465	418.5	-
Casein	200	140	126	200
Dextrinized cornstarch	132	155	139.5	-
Sucrose	100	100	90	499
Soybean oil	70	40	36	100
Lard	-	-	-	100
Fibre (Alphacel)	50	50	45	50
OFS	-	-	100	-
Mineral mix	35	35	31.5	35
Vitamin mix	10	10	9	10
L-Cystine	0.3	0.1	0.09	-
DL-Methionine	-	-	-	3
Choline bitartrate	2.5	5	4.5	2.5

The energy density of the control diet was 3.6kcal/g, the OFS diet was 3.4 kcal/g and the HFS diet was 4.6 kcal/g.

APPENDIX B: PCR PRIMERS

Table A.2. Primers used real time PCR in Chapters 4 & 5.

Gene	Forward Primer	Reverse Primer
B-Actin	TATCGGCAATGAGCGGTTCC	AGCACTGTGTTGGCATAGAGG
GPR 41	TGCTCCTCTTCCTGCCATTCC	CGTTCTATGCTCACCGTCATCAG
GPR 43	ACCCTCTGCTATTCTACTTCTCCTC	CCTCCACTGTCTCTTCGGCTC
AMPKα1	GCCCGACACACCTAGAT	TCCAACTGCTTGATTGCTCTAC

Primers are listed 5' to 3'

Table A3. Primers used for qPCR in Chapters 4 & 5.

Gene	Forward Primer	Reverse Primer
Total bacteria	ACTCCTACGGGAGGCAGC	GTATTACCGCGGCTGCTG
Bacteroides/ Prevotella	TCCTACGGGAGGCAGCAGT	CAATCGGAGTTCTTCGTG
Bifidobacteria	CGCGTCYGGTGTGAAAG	CCCCACATCCAGCATCCA
Methanobrevibacter	CTCACCGTCAGAATCGTTCCAGTC	ACTTGAGATCGGGAGAGGTTAGAGG
Enterobacteriaceae	CATTGACGTTACCCGCAGAAGAAGC	CTCTACGAGACTCAAGCTTGC
Lactobacillus	GAGGCAGCAGTAGGGAATCTTC	GGCCAGTTACTACCTCTATCCTTCTTC
C. coccoides	CAGCAGCCGCGGTAATA	CCCACACCTAGTAATCATCGTT
C. leptum	GCACAAGCAGTGGAGT	CTTCCTCCGTTTGTCAA
C. cluster XI	ACGCTACTTGAGGAGGA	GAGCCGTAGCCTTTCACT
C. cluster I	ATGCAAGTCGAGCGAKG	TATGCGGTATTAATCTYCCTTT
Roseburia	TACTGCATTGGAAACTGTCG	CGGCACCGAAGAGCAAT

Primers are listed 5' to 3'

APPENDIX C: SUMMARY OF DATA FROM ALL SEVEN MATERNAL PRE-PREGNANCY TREATMENTS

Table A4. Offspring Weight and Body Composition Data for all Seven Maternal Treatments

	Sex	Lean-CON	Control	OFS	Sitagliptin	OFS+S	CR	HFS-CON	Txt	Sex	Txt x Sex
11 week weight, g	M	415±37	417±32	411±27	409±63	430±40	423±42	401±33	0.6	<0.0001	0.7
	F	252±21	262±21	249±24	249±25	254±20	262±22	264±22			
Final weight, g	M	495±46	508±29	514±49	534±45	531±54	522±58	492±41	0.3	<0.001	0.2
	F	293±28	302±25	282±25	292±32	299±28	309±28	310±24			
Energy Intake, kcal	M	140±19[a]	148±48[ab]	153±43[ab]	169±58[b]	128±21[ab]	134±10[a]	150±24[a]	0.01	0.1	0.4
	F	123±15[a]	136±28[ab]	141±36[ab]	157±24[b]	145±36[ab]	134±17[a]	119±25[a]			
Body fat, %	M	15.8±4	21.8±5	18.2±5	21.7±7	22.1±5	22.6±9	21.2±8	0.03	0.8	0.9
	F	18.9±6	22.4±8	17.9±5	20.1±4	21.3±5	22.1±7	22.9±9			
Fat mass, g	M	79.3±25	110.8±28	94.8±35	118.0±47	118.9±33	112.4±53	106.3±51	0.05	<0.0001	0.6
	F	56.3±23	69.0±27	51.2±18	59.5±18	63.4±18	69.5±26	69.0±33			
Lean	M	416±32	374±81	419±34	416±30	412±37	400±36	389±35	0.3	<0.0001	0.1

mass, g	F	236±21	233±13	231±15	233±20	234±16	240±18	241±19			
BMD	M	0.165±0.004[ab]	0.163±0.004[a]	0.165±0.004[ab]	0.165±0.005[ab]	0.168±0.004[b]	0.166±0.005[ab]	0.165±0.005[ab]	0.01	<0.0001	1.0
	F	0.154±0.006[ab]	0.152±0.004[a]	0.155±0.004[ab]	0.154±0.005[ab]	0.158±0.005[b]	0.156±0.006[ab]	0.155±0.005[ab]			

Values are mean ± SEM (n=8-12 per group). [a,b]Values without a common superscript are significantly different within a sex ($P<0.05$).

Table A5. Offspring Fasting Blood Glucose and Satiety Hormone Levels Data for All Seven Maternal Treatment Groups.

	Sex	Lean-CON	C	OFS	S	OFS+S	CR	HFS-CON	Txt	Sex	Txt x Sex
Glucose at 11 weeks, mmol/l	M	5.7±0.2	6.2±0.2	6.2±0.3	5.9±0.1	6.2±0.3	6.1±0.2	5.6±0.1	0.4	0.1	0.1
	F	6.6±0.3	6.4±0.3	6.2±0.2	6.1±0.2	5.9±0.2	6.3±0.2	5.9±0.2			
Glucose at 17 weeks, mmol/l	M	6.0±0.2	5.8±0.2^{a}	5.8±0.1^{ab}	5.4±0.2^{b}	6.0±0.2^{ab}	5.9±0.2	5.7±0.3	0.1	0.04	0.7
	F	6.4±0.3	6.4±0.3^{a}	5.7±0.2^{ab}	5.6±0.1^{b}	6.0±0.2^{ab}	6.1±0.3	6.2±0.3			
Insulin, pg/ml	M	2222±854	1613±246	1936±356	1274±338	1783±389	1765±270	1125±205	0.7	0.002	0.6
	F	895±185	1174±321	1149±263	1290±488	1134±350	840±109	788±94.4			
Leptin, pg/ml	M	5450±1122^{a}	1985±449^{b}	5748±1015^{a}	2183±492^{ab}	5464±1303a^{b}	4368±860^{ab}	3782±814^{ab}	0.005	0.3	0.6
	F	5779±843^{a}	3286±954^{b}	5698±746^{a}	4854±974^{ab}	4669±817a^{b}	4637±1001^{ab}	3706±665^{ab}			
Ghrelin, pg/ml	M	178±30.0^{a}	146±35.5^{abc}	46.9±12.5^{b}	55.8±12.8^{c}	195±70.2^{ac}	181±32.6^{ac}	86±29.6^{abc}	<0.0001	<0.0001	0.4
	F	299±34.5^{a}	188±47.3^{abc}	117±16.1^{b}	82.2±29.0^{c}	186±25.8^{ac}	265±47.7^{ac}	194±31.2^{abc}			
GIP, pg/ml	M	106±31.3	36.6±7.8	58±16.2	37.9±6.3	55.8±11.9	42±9.14	43±14.4	0.06	0.002	0.8
	F	124±30.2	96.8±30.2	112±38.6	86.5±23.2	79.7±15.7	90±24.7	46±13.1			
PYY, pg/ml	M	29.5±2.8	26.8±6.1	23.8±5.7	25.8±4.4	39.4±11.9	32.4±4.4	28.1±5.8	0.9	0.1	0.6

	F	42.1±23	41.4±6.8	42.1±9.0	45.7±11.1	37.3±8.4	23.3±5.6	25.0±7.0			
GLP-1, pg/ml	M	11.4±3.0	11.5±2.9	9.8±2.5	10.3±2.5	11.2±2.6	9.5±2.9	10.5±3.3	0.6	0.8	0.7
	F	20.1±18	6.1±3.3	10.4±5.0	5.4±3.0	9.6±4.2	6.6±6.2	12.4±9.0			
HOMA-IR	M	15.8±6.2	10.2±1.6	12.1±2.4	7.3±2.1	11.7±2.4	11.3±1.9	6.7±1.4	0.5	0.003	0.5
	F	6.2±1.4	7.9±2.4	7.1±1.7	7.8±3.2	7.3±2.2	5.6±0.9	5.4±0.8			

Values are mean ± SEM (n=8-12 per group). [a,b]Values without a common superscript are significantly different within a sex ($P<0.05$).

Table A6. Offspring Gut Microbiota Data at 11 weeks for All Seven Maternal Treatment Groups.

	Sex	Lean-CON	C	OFS	S	OFS+S	CR	HFS-CON	Txt	Sex	Txt x Sex
Total bacteria	M	1528577±3E5^{abc}	1620403±2E5^{abc}	1114595±1E4^{a}	1660444±2E5^{abc}	1738225±4E5^{ab}	1439168±2E5^{abc}	2135845±2E5^{b}	0.5	0.4	0.01
	F	1622727±2E5^{abc}	1892998±9E4^{abc}	1742670±3E4^{bc}	1925955±1E5^{abc}	1892200±2E5^{abc}	1682758±2E5^{abc}	1173845±1E5^{ac}			
Bacteroides/	M	206348±5E4^{abc}	234669±4E4^{ab}	203351±3E4^{bc}	281381±3E4^{a}	182619±3E4^{abc}	162538±4E4^{bc}	150595±2E4^{c}	0.0001	0.5	0.7
Prevotella	F	221906±4E4^{abc}	247341±2E4^{ab}	193471±3E4^{bc}	331807±4E4^{a}	221109±6E4^{abc}	201099±2E4^{bc}	89843±2E4^{c}			
Bifidobacteria	M	57089±2E4^{abc}	16040±5E3^{ab}	6450±2E3^{a}	15080±5E3^{a}	32473±1E4^{ab}	87425±3E4^{b}	129036±4E4^{c}	0.0001	0.1	0.006
	F	38560±1E4^{abc}	34837±1E4^{abc}	5530±2E3^{abc}	32472±7E3^{abc}	51406±2E4^{abc}	46944±1E4^{abc}	33786±8E3^{ab}			
Methanobrevibacter	M	305±41^{a}	308±308^{a}	218±35^{a}	271±47^{a}	295±57^{a}	275±49^{a}	383±48^{a}	0.05	0.0001	0.04
	F	1134±123^{bd}	1630±256^{bc}	1149±154^{bd}	1154±153^{bd}	1179±95^{bcd}	1681±177^{c}	1090±162^{d}			
Enterobacteriaceae	M	1251±575	611±164	498±111	1296±319	1912±464	802±341	1134±303	0.4	0.2	0.4
	F	6510±4E3	393±103	611±137	430±58.6	1999±659	8482±7E3	1041±393			
Lactobacillus	M	414608±1E5	191678±4E4	212868±3E4	297918±5E4	166664±4E4	230522±6E4	261417±7E4	0.1	0.0001	0.2
	F	113912±3E4	149401±5E4	88966±4E4	250899±9E4	98519±3E4	148776±3E4	100695±2E4			
C. coccoides	M	1384961±3E5	1381770±3E5	1031245±2E5	930333±2E5	1198862±2E5	1092318±2E5	1206188±2E5	0.2	0.008	0.5
	F	1695949±2E5	1715540±3E5	1890208±4E5	1393081±2E5	1528047±1E5	1138887±2E5	1166514±2E5			
C. leptum	M	355277±2E5^{abc}	133726±5E4^{b}	95173±2E4^{b}	132884±3E4^{b}	101190±3E4^{b}	403136±1E5^{abc}	729532±2E5^{c}	0.3	0.001	0.0001

	F	629188±1E5[a]	496297±1E5[a]	545236±1E5[a]	726238±1E5[a]	470272±8E4[a]	304812±6E4[a]	313397±7E4[a]			
C. cluster XI	M	1841±557	1515±242	1595±284	2306±361	2287±326	2102±608	3664±617	0.05	0.0001	0.1
	F	456±74.1	608±80.7	698±353	977±499	823±232	516±57.7	696±168			
C. cluster I	M	11575±3E3[a]	8630±2E3[a]	9482±3E3[a]	7275±968[a]	16019±3E3[ab]	5602±2E3[a]	25129±5E3[b]	0.02	0.8	0.0001
	F	10269±2E3[ab]	13325±2E3[ab]	10444±3E3[ab]	14164±1E3[ab]	12824±3E3[ab]	11311±1E3[ab]	8520±2E3[a]			
Roseburia	M	64186±1E4[ab]	25769±6E3[ab]	19867±6E3[a]	25235±8E3[ab]	1689±437[a]	75538±3E4[b]	55271±2E4[ab]	0.04	0.0001	0.04
	F	323±133[c]	188±47.4[abc]	170±82.3[abc]	268±98.4[abc]	220±73.0[abc]	690±211[c]	263±118[c]			

Values are mean ± SEM (n=8-12 per group). [a,b]Values without a common superscript are significantly different within a sex ($P<0.05$).

Table A7. Offspring Gut Microbiota Data at 17 weeks for All Seven Maternal Treatment Groups.

	Sex	Lean-CON	C	OFS	S	OFS+S	CR	HFS-CON	Txt	Sex	Txt x Sex
Total bacteria	M	1223343±4E4	1787771±3E5	1794757±1E5	1447322±1E5	1628238±2E5	1691735±2E5	1735720±9E4	0.3	0.2	0.5
	F	1668852±8E4	1770551±2E5	1759542±2E5	1543517±9E4	1643443±6E4	3972506±1E5	3972506±2E6			
Bacteroides/	M	88449±9E3	120514±2E4	231077±6E4	184036±3E4	130359±2E4	211750±3E4	217888±4E4	0.3	0.09	0.7
Prevotella	F	202893±2E4	197086±3E4	205156±5E4	216999±2E4	199368±5E4	300308±5E4	558372±4E5			
Bifidobacteria	M	9012±3E3	5249±2E3	6395±2E3	6925±2E3	5850±2E3	9140±3E3	4620±3E3	0.1	0.2	0.7
	F	9552±4E3	7528±2E3	2043±687	1210±266	2854±572	7577±3E3	3372±1E3			
Methanobrevibacter	M	981±133	952±130	1214±154	1111±56.6	1142±254	1024±79.3	1222±113	0.8	0.4	0.8
	F	1119±181	934±94.4	924±138	1041±123	1124±175	974±97.4	1081±119			
Enterobacteriaceae	M	756±193	2352±699	747±125	1002±280	849±276	539±101	4009±3E3	0.7	0.9	0.4
	F	4335±4E3	1282±571	807±245	737±214	1133±284	1299±389	1107±455			
Lactobacillus	M	29007±9E3	46027±2E4	36547±1E4	117786±5E3	28509±1E4	8209±3E4	80211±5E4	0.3	0.4	0.3
	F	56272±3E4	66525±3E4	21871±7E3	21239±1E4	24996±8E3	27919±1E4	34664±1E4			
C. coccoides	M	924056±1E5	1683324±3E5	1757458±3E5	1567243±3E5	1621900±3E5	1479280±2E5	1796379±3E5	0.3	0.3	0.5

	F	1280902±2E5	1558111±3E5	641704±2E5	1431796±2E5	2083268±3E5	1421070±3E5	8267835±6E6			
C. leptum	M	535812±1E5	526511±1E5	1051284±2E5	956347±2E5	716135±2E5	799080±2E5	897344±2E5	0.1	0.3	0.2
	F	1102637±2E5	557425±1E5	1038521±2E5	746382±2E5	1026993±3E5	534553±7E4	1242820±3E5			
C. cluster XI	M	28373±1E4[a]	4426±1E3[ab]	6447±5E3[ab]	1071±263[b]	1159±293[b]	6193±3E3[ab]	5995±1E3[ab]	0.007	0.4	0.3
	F	9218±4E3[a]	5227±2E3[ab]	8191±4E3[ab]	514±217[b]	3005±938[b]	5689±2E3[ab]	4599±2E3[ab]			
C. cluster I	M	29359±2E4	7502±2E3	6407±1E3	4094±406	8061±2E3	8298±776	7702±2E3	0.08	<0.0001	0.06
	F	905±432	986±270	3140±2E3	399±132	1892±1E3	876±482	1065±653			
Roseburia	M	932±279	561±297	214±52.4	210±71.8	176±59.2	622±221	407±285	0.05	0.08	0.3
	F	294±116	511±207	188±52.0	165±54.6	241±66.6	293±73.6	329±108			

Values are mean ± SEM (n=8-12 per group). [a,b]Values without a common superscript are significantly different within a sex ($P<0.05$).

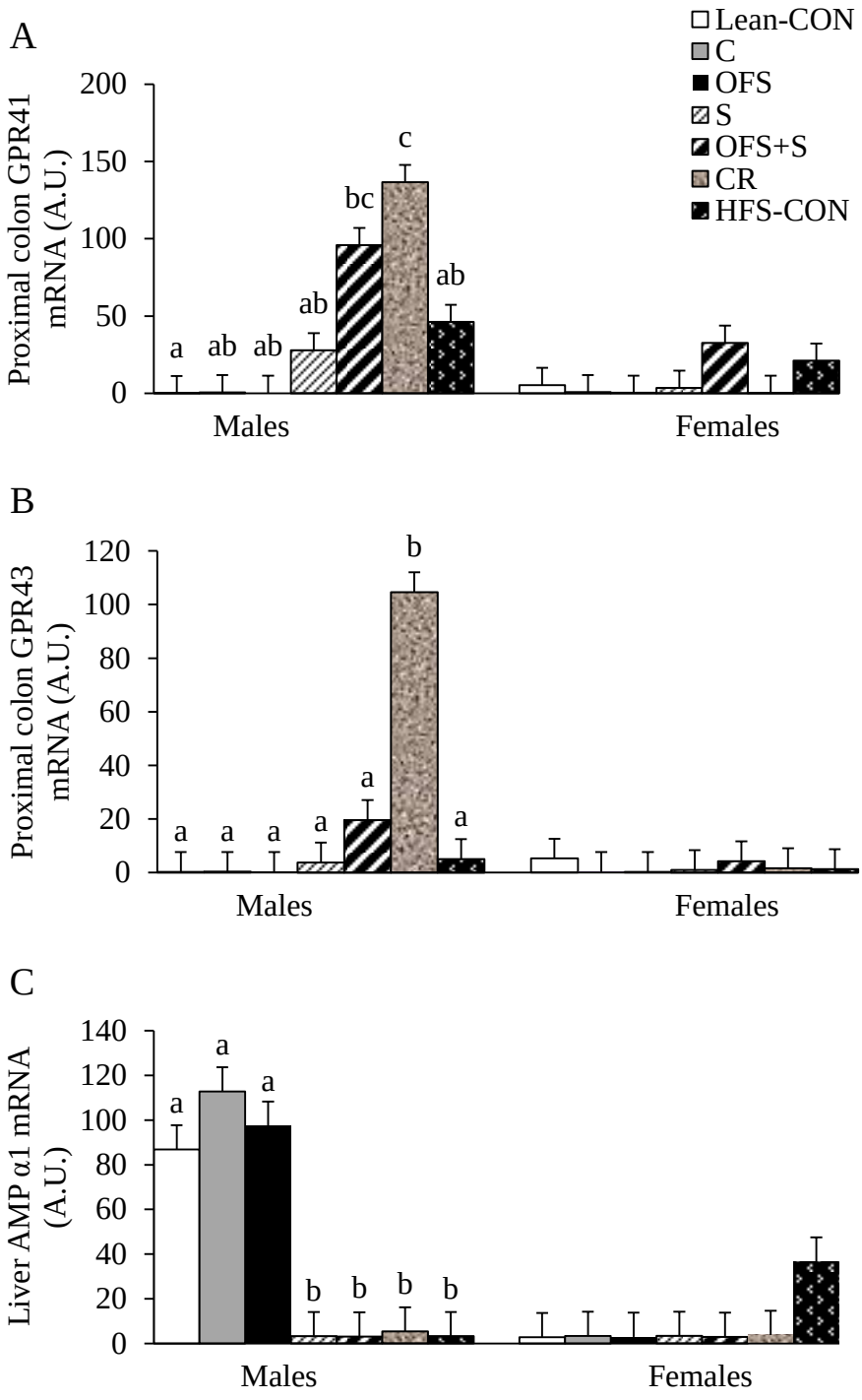

Figure A1. Offspring gene expression data for all seven maternal treatment groups.