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Prebiotic Fiber Supplementation Differentially Affects Metabolic Parameters Regulating Obesity in Rats Raised in Small versus Large Litters

by

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Abstract

The objective of this study was to examine weight gain and satiety hormone levels in rats raised in small versus large litters and weaned onto control or oligofructose (OFS)-enriched diets. Small litters (SL, 3 pups) and normal litters (NL, 12 pups) of male Sprague-Dawley rats were fed 1 of 4 diets from 3 to 19 weeks of age: 1) Normal Energy density (NE), 2) NE + 10% OFS, 3) High Energy density (HE), 4) HE + OFS. At weaning, SL body weight was higher than NL. OFS significantly reduced body weight independent of litter size. OFS significantly reduced glycemia in all groups except SL-NE rats and increased glucagon-like peptide-1 (GLP-1) in all groups except for NL-NE rats. Satiety hormone gene expression was differentially modified in SL and NL rats fed OFS. These results indicate that prebiotic fiber feeding was able to mitigate some aspects of programmed obesity risk.

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To Connal, thank you for always bringing out the best in me.

Dedication

To my family.

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List of Symbols, Abbreviations and Nomenclature

Symbol	Definition
ACC	Acetyl-coenzyme A carboxylase
AIN	American Institute of Nutrition
AMPK	AMP-activated protein kinase
ANOVA	Analysis of variance
ARC	Arcuate nucleus
AUC	Area under the curve
BMD	Bone mineral density
C	Control
CART	Cocaine- and amphetamine- regulated transcript
CD	Cluster of differentiation
CpG	Cytosine phosphodiester guanine
DEXA	Dual energy x-ray absorptiometry
DP	Degree of polymerization
DPP IV	Dipeptidyl peptidase 4
ELISA	Enzyme-linked immunosorbent assay
FAS	Fatty acid synthase
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GI	Gastrointestinal
GIP	Glucose-dependent insulinotropic peptide
GLP-1	Glucagon-like peptide-1
GPR	G-coupled protein receptor
HE	High energy
IUGR	Intrauterine growth restriction
mRNA	Messenger ribonucleic acid
MTT	Meal tolerance test
NE	Normal energy
NEFA	Non-esterfied fatty acids
NK	Natural killer
NL	Normal litter
NPY	Neuropeptide Y
OFS	Oligofructose
OGTT	Oral glucose tolerance test
POMC	Pro-opiomelanocorticod
PVH	Paraventricular hypothalamus
PYY	Peptide tyrosine tyrosine
RNA	Ribonucleic acid
rt-PCR	Real time-polymerase chain reaction
RYGB	Roux-en-Y gastric bypass
SCFA	Short chain fatty acid
SEM	Standard error of the mean
SL	Small litter
SREBP1c	Sterol regulatory element-binding protein 1
VLDL	Very-low-density lipoprotein

Chapter One: Introduction

1.1 Background

The alarming prevalence of obesity across the world has spurred a tremendous public health challenge (1). In 2005, it was estimated that 33% of the world's population 20 years and older was overweight or obese (2). It has been projected that by the year 2030, 57.8% of the adult population in the world will be either overweight or obese. Contributing to the obesity epidemic is the precipitous relationship between childhood weight gain and obesity in adulthood (1). Currently, it is estimated that globally more than 22 million children under the age of 5 are overweight with many developing countries encountering a doubling of the number of overweight children in the last two to three decades (3). The marked rise in number of overweight children has also contributed to an increase in comorbidities associated with insulin resistance and type 2 diabetes that were traditionally observed only in adult populations (3).

The consequences of the obesity epidemic are far reaching. Recently published data documenting the lifestyle habits of the Millennial Generation born between 1982-1993 showed that more than 75% of this generation does not eat the daily recommended number of fruits and vegetables and are at an increased risk of developing heart disease, hypertension, diabetes and a vast number of cancers (4). It has been projected that this generation may lose up to a trillion dollars in income due to obesity (4). Although this generation can be described as optimistic they are also plagued with being part of the childhood obesity epidemic (4). Not only is there concern for the health and well-being of these individuals but for the transgenerational increase in metabolic disease risk for future generations born to overweight and obese parents (5).

Current data suggests that preventing excessive weight gain is the key to curbing the obesity epidemic, however 10%-15% of children and adolescents are already overweight and obese (1). Therefore there is a need for researchers to develop novel interventions to treat and prevent the rise in obesity. Although it was previously thought that in humans, postnatal metabolic function was essentially developed at birth, recent evidence indicates that this period retains some plasticity, or the ability to respond to stimuli in the environment, and could become a target for reversing programmed obesity risk (6).

1.2 Significance

It was our objective to test whether or not dietary interventions, introduced early enough in postnatal life, were able to reverse any of the metabolic dysfunction that resulted from programmed obesity risk. Overnutrition during the suckling period is a well-defined model of programming that increases the susceptibility to obesity in rats (7). We have previously demonstrated favourable effects on energy intake and satiety hormone release of oligofructose in adult rats (8). Whether the same favourable response of the satiety hormones, GLP-1 and peptide YY (PYY), to oligofructose-rich diets occurs in young rats and persists into adulthood remained to be elucidated. By understanding the contribution of gut endocrine mediators of satiety to the potential reversal of programmed obesity, our ultimate goal is to combat childhood obesity that is increasingly prevalent in Canada.

1.3 Hypothesis

It was hypothesized that male Sprague-Dawley rats raised in small litters from birth to 3 weeks of age would have lower weight gain and improved gut satiety hormone profiles when weaned onto an oligofructose-enriched diet compared to control diet. Because of the complex nature of obesity, these physiological changes were expected to reduce some but not all of the increased susceptibility to obesity in later life.

1.4 Presentation

This thesis is comprised of the following chapters. Chapter one is a short introduction to the projects of this thesis and includes the hypothesis of the study. Chapter two is the literature review. Chapters three and four are manuscripts of the studies contained in this thesis. Chapter five is a discussion of the major findings of the work and suggestions for future investigations stemming from this initial effort. Appendix A contains graphical representations of the change from baseline (%) calculations for the satiety hormones and Appendix B includes significant correlations of body fat (%) with fasting plasma values and total AUC calculated values. Appendix C provides the limits of agreement graphs for the satiety hormone analysis and real-time polymerase chain reaction (rt-PCR). The primer sequences used for rt-PCR are listed in Appendix D.

Chapter Two: Literature Review

2.1 Developmental Origins of Health and Disease

2.1.1 Evidence from human studies

The Dutch famine occurring in the winter spanning 1944-1945 was the first documented and systematically evaluated occurrence of compromised nutritional status during intrauterine development leading to future obesity and heart disease risk in the offspring (9). In this cohort, the offspring of mothers who had been severely undernourished during the first trimester of pregnancy were found to have higher rates of obesity in adulthood compared to female offspring exposed to undernutrition in the second or third trimesters. Since the publication of this initial observation considerable research has been conducted on the link between intrauterine nutritional exposure and the development of obesity (10, 11).

First presented by Hales and Barker in 1992, the "thrifty phenotype hypothesis" was proposed after it was observed that children born small for gestational age due to intrauterine growth restriction (IUGR) were at a higher risk of developing dyslipidaemia and hyperinsulinaemia compared to children who had developed in a nutritionally-balanced environment (12). It was postulated that during prenatal growth the fetus adapts its tissue and endocrine system development to the undernourished environment which it predicts will remain similar after birth. A variety of changes have been observed in this scenario including alterations to gene expression, irregular metabolic processes and disruption of normal energy homeostatic mechanisms including increase body fat stores, symptoms of metabolic syndrome and increased risk for cardiovascular disease (11).

When the child is born into an environment that offers abundant calorie-dense foods that do not match its intrauterine environment, metabolic systems that have not been programmed to manage excess energy fail and excessive weight gain ensues. Malnourishment during intrauterine growth was an initial scenario of adverse dietary programming and has been used to help further understand metabolic homeostatic mechanisms (13, 14). However, prenatal influences such as IUGR and resulting childhood obesity have been identified as one of the many triggers of pediatric obesity. Postnatal nutritional status and resulting growth velocity also warrants attention (15).

In the past, children who were born premature or small for gestational age were provided with extra nutrition to accelerate their growth to match babies born at normal gestational age and weight (7, 16, 17). Unfortunately follow-up studies of these children have linked low birth weight and accelerated early growth with higher adult weight and increased susceptibility to cardiovascular disease (18). In a large American multi-center cohort study it was found that rapid weight gain during the first four months of life led to a greater chance of being overweight in childhood (19). Of particular note, this cohort was comprised of babies born full term and in the healthy range for gestational weight. A strong correlation was also found between the children who were overweight at age 7 and had the greatest weight gain in the first four months of life. Although this study did not follow the specific growth rate of the children over the four months they had a large representative cohort of children. Future work is required to prospectively assess the relationship between infant and childhood weight gain and future disease risk.

As the high rates of obesity now cross multiple generations, research is increasingly aimed at attenuating the development of obesity in infants and young children before it

tracks into adulthood (4). Because of ethical constraints in human research in this area, the well-established oversuckled rat model has been used to quantify changes in key gastrointestinal hormones controlling appetite and body composition following specific dietary interventions (11).

2.1.2 Evidence from animal studies

In order to understand more about the deleterious effects of postnatal overfeeding, rat models have been widely used (20-25). It has been shown that manipulating litter size after parturition programs rats to either a lean phenotype (raised in a large litter) or obese phenotype (raised in a small litter). Work by the Hahn laboratory is one of the first examples of this type of programming (21). Using the litter manipulation model in mice and rats he found that animals in a litter size of 4 compared to 14 developed high insulin levels when fed either a regular chow diet or high-fat diet. To assess blood pressure, neural-hormonal changes and adiposity markers, Velkoska et al. reduced litter sizes to either 3 or 12 male rat pups at birth then randomized the pups to either a high-fat diet or standard chow at weaning (24). When these rats were followed into adulthood, the rats that received overnutrition during the suckling period were more apt to develop hypertension, dyslipidaemia, obesity and insulin resistance. Plagemann et al. reduced litter size to only three pups per dam and found increased plasma insulin and leptin levels as well as increased body weight and food intake in these animals (15). In addition to studying some of the hormone changes present in the oversuckled animal, researchers have also adjusted the macronutrient composition of milk provided to the animals to help elucidate the specific triggers that may be responsible for metabolic changes in these animals (26).

2.1.3 Programming by changes in dietary macronutrient composition

Carbohydrates are one of the macronutrients that have been manipulated to induce negative metabolic consequences (26). Exchanging high fat rat milk for a formula high in carbohydrates resulted in hyperinsulinaemia in the pups within 24 hours. Analysis of the pancreatic islet cells isolated from rat pups fed a high carbohydrate formula for 12 days showed an increased secretion of insulin when challenged at various levels of glucose concentrations (27). In addition to increased expression of preproinsulin genes coding for insulin production there was also a significant reduction in islet cell size and an increased density of small-sized islet cells. Leptin production was also blunted in these animals. Central nervous system alterations that have the potential to change metabolic homeostatic control have also been observed after 12 days of high carbohydrate diet treatment (28). Hypothalamic increases in the orexigenic pathways such as neuropeptide Y (NPY) and decreased mRNA levels of the anorexigenic pro-opiomelanocortin (POMC) and cocaine-and-amphetamine-regulated transcript (CART) predisposed rats to obesity in adulthood (20, 24). Further work to fully understand the hormonal and neural changes with developmental programming and their consequences is needed.

2.2 Epigenetic Control of Metabolism

2.2.1 Epigenetic mechanisms

At this point there is substantial evidence to indicate that differences between intrauterine and postnatal environments can lead to increased susceptibility to weight gain (28). What remains unclear is the underlying mechanism(s) that cause these changes (17). Through epidemiologic evidence and studies using animal models critical periods

during development have been identified to be more susceptible to the programming of chronic disease (17). Epigenetics is the term used to describe the heritable potential of mitotic and possibly meiotic alterations in gene expression not caused by changes in DNA sequences (29). Under the general concept of epigenetics, "metabolic imprinting" has been adapted as the term to describe the effects of nutrition during these critical periods of development (30).

One such mechanism of epigenetic programming is the process of cytosine methylation. This process has been compared to packing 10,000 miles of cooked spaghetti, the entire human genome sequence into a basketball, or the nucleus of the cell (30). In essence, the complete DNA sequence is condensed using histone proteins into packages called chromatin. The two types of chromatin are either the chemically inactive form known as heterochromatin or the transcriptionally active euchromatin. The parts of the chromatin that allow tissue-specific transcription include the cytosines that are methylated within cytosine-phosphodiester-guanosine (CpG) dinucleotides and autoregulatory DNA-binding proteins (31). The CpG dinucleotides are methylated to 5-methylcytosine, which plays a key role in moderating the transcription of certain genes by modifying the properties of DNA-binding proteins. Upon fertilization, the CpG methylation of both the sperm and egg are almost but not completely erased and a new process of methylation begins as early as implantation and continues into the postnatal period (30, 31).

2.2.2 Nutritional influences on epigenetic programming

CpG methylation has been specifically highlighted due to the fact that nutritional factors such as folate, vitamins B_6 and B_{12} as well as choline and methionine act as

methyl donors and cofactors in the methylation pathway (30). As prebiotic fiber, discussed in greater detail below, can increase vitamin and mineral absorption, this dietary intervention may play a direct role in DNA methylation (30). Changes to methylation exerted via moderate shifts in nutritional environment highlight the delicate nature of developmental periods and provides clues for why the intrauterine nutritional environment plays such a crucial role in tissue development (17). One tissue that is particularly susceptible to the nutritional environment is the gastrointestinal (GI) tract (30). The GI system is the largest endocrine organ in the body and plays a direct role in controlling food intake and nutrient absorption (32). Studying the expression of specific genes from the gastrointestinal tissue in young rats exposed to diets varying in macronutrient content may help to elucidate some of the factors behind the development of obesity.

2.3 Enteroendocrine Physiology

The GI system responds to the ingestion of food particles by releasing a variety of hormones (33). These hormones have in common the ability to regulate food intake and energy metabolism and as a result are known as satiety hormones (33, 34). Certain hormones of interest include GLP-1, PYY, ghrelin and leptin (adipose-derived). It has been observed that these hormones play a direct role in satiety by binding to specific receptors in the target tissue in response to the macronutrient(s) ingested (33). Secondly, it is thought that these hormones interact with the central nervous system to relay to the body information regarding nutrient intake and energy status (34).

2.3.1 Glucagon–like peptide-1

The actions of the satiety hormone glucagon-like peptide-1 and glucose-dependent insulinotropic polypeptide (GIP) to a lesser extent have been studied due their potential to become a treatment strategy for obesity (33). Produced by abundant L cells in the distal ileum and colon, the action of GLP-1 is associated with glucose homeostasis, delayed gastric emptying and a prolonged sense of fullness (35). Gut hormones such as GLP-1 and GIP are categorized as incretins due to their ability to increase insulin secretion following oral nutrient intake compared to intravenous glucose administration (33). This response indicates that glucose-sensing mechanisms may be located on the luminal side of the intestine (33, 34). Furthermore, it should be noted that GLP-1 has been detected in hepatoportal blood as soon as nutrients arrive in the duodenum before they reach the distally located L cells and therefore neural regulation has also been implicated (33).

It has been determined that the release of GLP-1 is under both hormonal and neural control yet the specific mechanisms have not been clearly identified (36). Even though GLP-1 is released in the distal small intestine, circulating levels of the hormone can be identified long before glucose or other nutrients reach the ileum (33, 34). A study by Wishart et al. using 12 healthy volunteers followed the rate of gastric emptying and GLP-1 release after the ingestion of a sugar-water solution and technetium-sulphur colloid (37). It took an average of 115 ± 11 minutes for 50% of the stomach contents to empty but only 30 minutes for GLP-1 to reach peak plasma levels. The inverse relationship between an increased release of GLP-1 and decreased gastric emptying is unique to GLP-1 and requires further inquiry (38). Evidence for a role of GLP-1 in

physiological satiety exists, however, further insight into the specific mechanisms controlling the release of this hormone may allow researchers to better mimic its positive effects on glucose homeostasis and delayed gastric emptying.

Following consumption of protein, carbohydrates, fat or mixed meals, active GLP-1 circulates for only a short period of time (<2 min) due to the rapid enzymatic degradation by dipeptidyl peptidase IV (DPP IV) (33). Consequently, it has been proposed that the receptor for GLP-1 is located close to the secretory cells (33, 34). GLP-1 interacts with the brain by diffusing across the blood-brain barrier into the hypothalamus as well as via afferent innervation of the vagus nerve in the gastrointestinal system (33). GLP-1 infusion has been suggested as a possible treatment for obesity given the observation that GLP-1 response is often diminished in human obesity (39). The administration of intravenous GLP-1 to obese subjects before a meal led to slower gastric emptying, a prolonged sense of fullness and a lower postprandial glucose response (39). The encouraging results observed with direct infusion of GLP-1 are limited due to the rapid degradation of the incretin, the impractical nature of continuous intravenous administration and the gastrointestinal upset reported with GLP-1 agonists (40). As well, the long-term effects and safety of the various treatment forms have yet to be elucidated.

Although researchers are continuing to develop alternative pharmacological treatments that mimic the effects of GLP-1, endogenous stimulation of GLP-1 via non-digestible carbohydrate dietary interventions is also being considered (41). Encouraging data linking the consumption of dietary fiber with both hyperplasia and hypertrophy of L cells and increased GLP-1 secretion has added support for this strategy. In depth discussion of the role of prebiotic fibers in stimulating endogenous GLP-1 release is

provided below. Future research is needed to build a strong evidence base for the stimulation of endogenous GLP-1 secretion in addition to pharmacological treatment options.

2.3.2 Peptide tyrosine-tyrosine (PYY)

As another gut hormone released by L cells, PYY is secreted in direct proportion to the calories ingested and is thought to decrease gastrointestinal motility, regulate insulin secretion and is involved in glucose homeostasis (42). Approximately 60-70% of PYY (1-36) is converted into the active form of PYY (3-36) by DPP IV and acts on the specific human receptor Y2 in the hypothalamus (42, 43). Upon ingestion of a meal, PYY binds to the hypothalamic Y2 receptor to reduce further food intake by inhibiting NPY neurotransmitters in the arcuate nucleus (ARC) of the hypothalamus (44). Damage to the vagal afferent pathway from the gut to the brain also inhibits the natural reduction in food intake attributable to PYY (42).

It is interesting to note that numerous studies have indicated that a high dietary intake of protein elicits greater circulating levels of PYY compared to carbohydrates and lipids (42). A study by Batterham et al. used a randomized crossover design with normal-weight and obese male subjects to assess the PYY response to various isocaloric meals. In both the normal-weight and obese subjects, protein was found to be the most satiating and have the greatest effect on hunger modification (43). Boey et al. (45) and Le Roux et al. (46) have published similar results of increased satiety and report heightened PYY secretion upon high protein feeding. In addition to the short-term satiating effects of a high protein meal, studies using mice models fed high-protein/high-fat diets or high-protein/low-fat diets for 16 weeks resulted in equivalent reduction in food intake and

weight loss (43). Both human trials and rodent models have demonstrated a secretory effect of protein on PYY yet the connection between macronutrient consumption and resulting PYY secretion are not well understood at this point nor are the actions of PYY on the central nervous system (43, 46).

The discovery of the two forms of PYY and the peripheral and central actions of this hormone make it a focus for obesity research as well as a challenging satiety signal to study (42). It is key to acknowledge that PYY injected directly into the brain leads to increased food intake, a result completely opposite to reduced food intake observed with peripheral treatment. As well, contradicting results from rodent studies and human studies looking at similar exposure variables limit the ability to generalize findings across species. Continued research looking at PYY secretion in response to changes in diet will ultimately contribute to further understanding the actions of this hormone.

In obese patients, both resting and postprandial responses of PYY are diminished and therefore intravenous injections of this hormone have been targeted as a pharmacological treatment for obesity (44). Exogenous therapy of PYY has been shown to induce mild weight loss in obese subjects but further testing of this agent was halted due to the development of nausea in the subjects (42). The observed decrease in circulating levels of PYY present in obese subjects has made it a popular target for obesity treatment (43). However, negative responses from human trials and conditioned taste aversion in rat studies has somewhat lessened the enthusiasm for this treatment form (47). PYY continues to be an intriguing yet controversial target that needs further research in order to clearly determine its physiological actions in humans.

2.3.3 Ghrelin

The discovery of ghrelin as one of the most powerful orexigenic (increases food intake) and adipogenic peptides in the human body led to an increased understanding of the link between the peripheral and central control of energy balance (48). The main site of production of ghrelin is the stomach but the presence of ghrelin-releasing cells has been detected as far down the intestinal tract as the colon. The actions of ghrelin have been related to various functions including appetite stimulation, control of gastric motility and growth hormone secretion as well as having influence over sleep, the cardiovascular and immune systems. The relationship with ghrelin and the release of growth hormone from the pituitary gland in the brain helped determine its chief function as an endogenous ligand for the growth hormone secretagogue receptor (now thought to be a g-protein coupled receptor) (49).

This metabolically active neuroendocrine hormone plays a role in almost every part of the human body (50). Studies in humans involving prolonged fasting show increased levels of circulating ghrelin that then decrease to basal levels approximately one hour following food consumption (48, 49). The effect of ghrelin is mediated by glucose or meal intake and not necessarily due to a response in insulin release or gastric distension (49, 50). Compared to lean controls, obese subjects have been found to have lower resting concentrations of ghrelin and the characteristic decline in ghrelin post-meal is lacking such that ghrelin exposure would be greater throughout the day compared to normal weight individuals (50). Circulating levels of ghrelin typically increase after weight loss and decrease with weight gain (49). In children, it has also been shown that levels of ghrelin decrease with increasing age (48). There appears to be a link between

energy homeostasis and appropriate growth and reproduction. Considering the complex network in which ghrelin operates in the human body further research is required to understand its full physiological activity.

2.3.4 Leptin

Similar to the decreased secretion of GLP-1 in obese individuals, atypical leptin responses have been observed in individuals who are obese (51). The *ob* gene which codes for leptin is found in white adipose tissue, the stomach, placenta and possibly the mammary gland (52). This anorexigenic peptide hormone has been observed to be produced proportionally as body fat content increases (51). Leptin resistance has also been linked with the impaired secretion of GLP-1.

Leptin has been related to infant development due to the fact that it is present in breast milk and markedly absent in infant formula (53). Given that leptin is one of the main regulators of food intake and the observation that breastfed and formula fed infants differentially self-regulate milk consumption, it has been proposed that a lack of dietary leptin may contribute to the higher rates of obesity development in formula-fed infants. Due to the peptide structure of leptin, additional research is required to quantify the amount of leptin that is absorbed into the blood stream after digestion once it has been passed to the infant from the mother. It has also been suggested that increased body fat deposition in formula fed infants leads to slight metabolic set point differences and fat oxidation rates between obese and non-obese individuals (52). Assessing leptin levels of animals that were exposed to high intakes of nutrition during the suckling period may have lasting effects that persist into adulthood (15). Characterizing changes in leptin sensitivity may help to understand the patterns of metabolic imprinting.

Another satiety hormone, amylin is often discussed in relation to leptin (54). Secreted from pancreatic islet cells in response to meal consumption, amylin interacts with neurons in the area postrema of the hypothalamus to decrease meal size. In addition to acting as a short-term anorexigenic hormone, amylin displays long-term adiposity signalling properties similar to insulin and leptin. Parallel to leptin resistance in people who are obese, higher levels of amylin have been found in an obese state compared to lean controls. Current research is aimed at understanding more of the whole body actions of this hormone including its' role in the development of the brain stem as well as the use of amylin in conjunction with leptin as a therapeutic agent in treating obesity.

2.4 Bariatric Surgery

At this point bariatric surgery involving physical reconstruction of the stomach and/or small bowel is recognized to be the most effective long-term treatment for obesity and related comorbidities. (55). The Roux-en-Y gastric bypass (RYGB) procedure accounts for approximately 65% of all bariatric surgeries and involves the creation of a small stomach pouch and anastomosis of the upper duodenum with the distal jejunum, bypassing the majority of the duodenum and proximal jejunum (56). While this treatment reduces caloric intake and nutrient absorption, adjusting the anatomy of the upper gastrointestinal system has also been found to lead to changes in the secretion of satiety hormones including GLP-1, PYY and ghrelin. It has been suggested that malabsorption of calories may or may not contribute to the considerable weight loss observed in patients following RYGB surgery however a substantial increase in post-prandial GLP-1 and significant increases PYY as well as satiety ratings have been associated with strong

responders to RYGB versus poor responders (55, 57). In a study comparing RYGB surgery to a diet and exercise plan, similar weight loss was achieved between groups however only significant increases in PYY and GLP-1 and a reduction in appetite were observed in the RYGB group further suggesting that increased levels of GLP-1 and PYY may play a key role in enhancing weight loss following this procedure (57). Although the literature is mixed, it appears that ghrelin levels decrease in patients following a RYGB procedure or in some cases do not change adding to the evidence that changes in satiety hormones following bariatric surgery contribute to the success of this treatment plan (56). The long-term weight loss success rate of bariatric surgery options makes this approach attractive however the massive lifestyle change required to succeed with this treatment as well as the cost of the surgical intervention limits its wide-spread usage (58). Instead alternative methods to increase satiety hormone secretion including dietary supplementation paired with other lifestyle modifications including satiety hormone analogues may lead to successful weight loss and a reduction in obesity-related comorbidities.

2.5 Prebiotic Fiber

As the direct pharmacological treatment of obesity with satiety hormones has proven difficult in humans due to adverse side effects, alternative treatments that trigger endogenous secretion of these hormones, such as dietary fiber have been proposed (59). From the family of fructans characterized as carbohydrates with one or more fructosyl-fructose osidic bonds, fiber sources such as oligosaccharides have been studied due to their unique digestive properties. Specifically, inulin and oligofructose are long and

short-chain fructo-oligosaccharides respectively that are derived from the chicory root and commonly added into food products. Inulin and oligofructose share a very similar structure but can be differentiated because oligofructose has a shorter chain length (60). Molecules with lower degrees of polymerization (DP) such as oligofructose are faster and more completely fermented compared to inulin. Now classified as prebiotics, these types of fiber are unique because they resist digestion in the upper gastrointestinal tract and are preferentially fermented by health-promoting bacterial strains in the colon (61). The breakdown of this fiber does not lead to glucose and fructose but instead short chain fatty acids (SCFA), lactate and gases produced by anaerobic bacterial fermentation.

Oligosaccharides have become attractive to study because they are a source of soluble fiber which are water-soluble, non-viscous and contribute positive organoleptic properties (taste, mouth-feel) to food without the accompanying calorie increase (59). Inulin and oligofructose have an estimated caloric value of ~1.5 kcal/g compared to the 4 kcal/g of digestible carbohydrate (61). Other beneficial effects include delaying gastric emptying, improving glucose and insulin responses, boosting the immune system, protection against certain cancers, increasing the absorption of calcium and stimulating the release of certain satiety hormones (61, 62). These prebiotic fibers have been used in certain populations to aid in weight loss (63). After consumption of oligofructose twice daily for two weeks there was a marked decrease in food intake and an increase in GLP-1 secretion in normal weight human subjects (63). Furthermore, our lab has recently demonstrated significant weight loss in overweight and obese adults consuming an oligofructose supplement for 3 months versus placebo treatment (64). Although some subjects have experienced uncomfortable side effects following prebiotic fiber

consumption these effects may be transient and can be lessened with a reduction in the amount of daily fiber consumed.

Numerous mechanisms have been proposed to explain how prebiotic fiber assists in weight loss and improves metabolic function. In normal and obese Zucker rats, decreased liver fat accumulation was observed with fructo-oligosaccharide treatment (59). It is suggested that due to the fermentation of oligosaccharides in the distal intestine certain SCFA that inhibit hepatic lipid synthesis (i.e. butyrate and propionate) are produced in greater quantities than lipogenic SCFA's (i.e. acetate). Although there was no observed change in postprandial triglyceridemia in obese Zucker rats, some human studies supplementing fermentable oligosaccharides of doses ranging from 8 to 20g/day have reported reduced serum triglyceride levels (62). Further study into the relationship between SCFA production and reduced hepatic lipid storage is warranted.

Related to colonic fermentation of oligosaccharides by bifidobacteria, increased absorption of certain minerals such as calcium and magnesium has been observed in rats (60). As both vitamins and minerals play key roles in enzymatic reactions involved with metabolism, increased absorption of micronutrients has been shown to affect biochemical processes related to appetite regulation and insulin sensitivity and help with body weight loss and management (65).

2.6 Reversal of Dietary Programming

Allowing rat pups access to excessive amounts of nutrition has the ability to directly modify the metabolic functions of these animals similar to changes seen in overweight and obese children (7). However, to date, there has been limited research

published attempting to reverse the effects of early postnatal dietary programming with a subsequent dietary intervention (66). We have previously demonstrated that rats consuming a high fiber diet from weaning into adulthood were protected against excess adiposity following a high fat/high sucrose diet challenge, but whether or not this same protection can be imparted after overnutrition during the suckling period remains to be elucidated (6).

The metabolic regulatory effects of the prebiotic fiber coupled with the critical period of postnatal development suggest that a diet enriched with oligofructose may be able to reverse some of the effects of obesity programming via certain interrelated mechanisms (30, 59). The ability to increase certain types of SCFA production with the fiber-supplemented diet may contribute to slower body weight accumulation and increased secretion of satiety hormones (59). Our recent demonstration that weaning onto a high fiber diet was protective against weight gain when challenged with a high-fat high-sucrose diet in adulthood suggests this dietary strategy may effectively target other time points that influence long-term susceptibility to obesity (6). It was also found that rats on the high fiber diet had smaller livers and increased colon weight compared to high protein and control diet groups (6). This suggests that there may be a powerful interaction between the stimulus of the products of prebiotic fiber fermentation and a feedback mechanism to control satiety (59). It is our suggestion that predisposing rat pups to obesity during the suckling period and subsequently exposing them to prebiotic fiber immediately after weaning may promote a protective effect for future obesity development and potentially reverse some of the effects of the dietary programming.

2.6.1 Evidence of reversing programming

To date the only documented effective intervention for reversing adverse dietary programming used leptin administration to normalize growth of undernourished rats (66). Evidence from the literature indicates that restricting calories during *in utero* development programs rat pups to gain excessive amounts of weight in adulthood especially when challenged with a high-fat diet. Based on those findings a study was completed to compare the ability of leptin to reverse obesity development in undernourished rat pups (66). Undernourished and ad libitum fed pups were treated with either saline or recombinant rat leptin for 10 days after birth. After feeding both the undernourished pups and calorie-sufficient pups a high fat diet, it was shown that the undernourished rats treated with leptin injections had equivalent body weight to pups exposed to sufficient nutrition during *in utero* development. This study provided for the first time evidence to suggest that *in utero* dietary imprinting was not necessarily irreversible as previously thought (30, 66). Although it must be acknowledged that there are some differences in developmental critical periods between rats and humans, this initial evidence provides the opportunity to take advantage of the plasticity during these periods to potentially reverse programmed obesity (30, 59).

2.7 Rationale

The focus of this research was a two-pronged approach to preventing obesity that involved identifying whole body responses to dietary fiber supplementation and identifying the biochemical mechanisms at the cellular level that bring about these changes. Ultimately the insight gained from this study will be returned to the community

for clinical application in preventing pediatric obesity. In targeting the early metabolic function and life-long programming of young rats, this novel research aimed to demonstrate that the dietary fiber, oligofructose, could be used as a primary prevention strategy in managing pediatric obesity.

As inulin and oligofructose are relatively new dietary treatment options, further research is needed to see if longer treatment intervals continue to elicit the same beneficial effects (61). Infant formula that has been fortified with inulin increased the growth of bifidobacteria in formula-fed infants that matched the fecal content of bifidobacteria in breast-fed infants (67). Given that certain commercial infant formulas and numerous weaning foods now contain prebiotic fiber, it was imperative that the effects of this inclusion be examined beyond changes in gut microbiota and include outcomes such as body composition and energy intake from childhood into adulthood. Continued research into the daily consumption of prebiotic fiber is warranted in both child and adult populations, especially to quantify long-term endogenous hormone secretion and the stability of the gut microbiota profile.

The mechanistic aspects of this research targeted an increase in our understanding of the role of prebiotic fiber, via alterations in gut satiety hormones and hepatic lipid metabolism, in preventing obesity. Overnutrition during the suckling period is a well-defined model of programmed susceptibility to obesity in rats (15, 16, 21, 24). The goal of this work was to evaluate the ability of oligofructose fiber introduced at weaning to reverse or mitigate the increased susceptibility to obesity brought about by litter size manipulation. Use of the rat model was the first step in quantifying the usefulness of prebiotic fiber in the diet which may provide concrete evidence for the integration of this

type of fiber into daily life. By understanding the contribution of gut endocrine mediators of satiety to reversed programming of obesity, our ultimate goal is to combat childhood obesity that is so prevalent in Canada.

Chapter Three: Oligofructose reduces body fat and glycemia to a greater extent in rats raised in large versus small litters[1]

3.1 Introduction

Unprecedented obesity rates in children and adults are dramatically changing the burden of non-communicable disease worldwide (1). Of present concern is the trajectory of childhood adiposity developing into obesity in adulthood (2). Similar to adults, overweight and obese children are at increased risk of developing comorbidities associated with insulin resistance and type 2 diabetes (3). There is a growing consensus that preventing excessive weight gain in childhood, and even as early as infancy, is a key target for curbing the obesity epidemic (1).

Critical periods of growth and adiposity development surrounding pre and postnatal periods have been recognized for their relationship to epigenetic changes in metabolism (7, 28, 68). Rodent models imposing maternal undernutrition typically result in pups that are overweight and hyperinsulinemic in adulthood, although variations in the severity of these outcomes exist (7). Although the deleterious effects of dietary malprogramming have been characterized, limited research to date has evaluated whether or not these programmed defects can be reversed. In one case, postnatal leptin injections were able to prevent excessive weight gain in rat pups that had been undernourished *in utero* (59).

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[1] A version of this chapter has been submitted. Reid DT, Reimer RA. *Oligofructose reduces body fat and glycemia to a greater extent in rats raised in large versus small litters.* Submitted Oct. 4, 2010 to Obesity. This work was presented in part at the International Conference on Developmental Origins of Health and Disease, May 6-8, 2010, Munich, Germany. Reid DT, Reimer RA (2010) Use of oligofructose to regulate gut satiety hormones and prevent obesity in young rats.

Based on this finding it was suggested that postnatal development retains a higher degree of plasticity than originally thought and novel strategies to reverse adverse dietary programming have become an attractive target for obesity intervention.

Dietary modification such as supplementation with prebiotic fiber is one such potential strategy (52). We have recently shown that feeding a prebiotic fiber-enriched diet to rats from weaning into adulthood was able to protect offspring from rapid weight gain, excess body fat and hyperglycaemia when challenged with a high fat/high sucrose diet in adulthood (6). Adult rats fed prebiotics responded to the diet by decreasing food intake, in part due to increased satiety hormone release. Oversuckling rats from birth to weaning, achieved by decreasing litter size, is one model that demonstrates long-term metabolic changes due to early postnatal overnutrition (24). Whether or not prebiotic fiber, when introduced at weaning, is able to mitigate some of the detrimental metabolic programming associated with oversuckling is not known.

Using the oversuckled rat model our objective was to determine if a diet supplemented with prebiotic fiber could attenuate programmed susceptibility to obesity and hyperglycemia due to postnatal overnutrition. Specifically, we examined the response of satiety hormones and tissue-specific gene expression in adult rats exposed to oligofructose (OFS) diets from weaning to 19 weeks of age. It was hypothesized that supplementing the diet of oversuckled rats with OFS would be associated with a more favourable satiety hormone profile compared to rats fed a control diet.

3.2 Methods and Procedures

3.2.1 Animals and housing

The study protocol was approved by The University of Calgary Animal Care Committee and conformed to the *Guide for the Care and Use of Laboratory Animals*. Twenty first-time pregnant Sprague-Dawley female rats were obtained from Charles River (St. Constant, QC) and kept on a 12-hour light-dark cycle in a temperature and humidity controlled room in the University of Calgary Animal Resource Center. Rats were approximately one week pregnant at the time of arrival in our facility.

One day after birth, litters were randomized to become small litters (SL, culled to 3 male pups) or normal litters (NL, culled to 12 pups including both males and females). Decreasing litter size to 3-4 pups at birth and throughout the suckling period is associated with overnourishment and results in rats that are obese, hyperglycaemic and insulin resistant compared to rats raised in normal litters (10-12 pups in size) (24). At weaning, male rats were randomized into the experimental diet groups (9-10 rats per group) and housed in pairs for the duration of the study. Each SL rat group was formed by 6-7 different litters and each NL rat group was combined from 4-5 different litters.

3.2.2 Diets

Standard control growth (AIN-93G) and maintenance (AIN-93M) diets were obtained from Dyets Inc (Bethlehem, PA). Oligofructose (Raftilose P95), a prebiotic fiber with a degree of polymerization of 4.5, was provided by Quadra Chemicals (Vaudreuil-Dorion, PQ, Canada). All diets met the nutritional requirements for rats during growth (up to 10 wk of age) or maintenance of adult rats (10 wk onwards). At weaning, control (C) diet rats received AIN-93G (3.8kcal/g) and at 10 weeks of age were switched onto

AIN-93M (3.6kcal/g) for the remainder of the study. OFS-supplemented diet contained 10% OFS by weight and had 3.5kcal/g and 3.4kcal/g for growth and maintenance diets, respectively. The OFS diet was prepared in house by mixing 90 g of AIN-93 diet with 10 g of OFS. Food and water was provided *ad libitum* and food intake measurements made throughout the study. Food intake was measured by weighing the difference in feed cups from one day to the next accounting for any spillage. Food intake was recorded over a week for a total of 7 independent weeks throughout the study (approximately every 2 weeks).

3.2.3 Body weight and composition measurements

Body weight was recorded using an electronic scale on the same day of every week for the duration of the study. One day before sacrifice, under light anaesthetic (Isoflurane) lean mass, fat mass and bone mineral density (BMD) was measured via dual energy x-ray absorptiometry (DEXA) using software for small animals (Hologic QDR 4500, Hologic, Inc., Bedford, MA).

3.2.4 Oral glucose tolerance test (OGTT)

One week prior to sacrifice an OGTT was performed to assess blood glucose response. Following an overnight fast, blood was sampled via tail nick followed by an oral glucose gavage (2 g/kg). At 15, 30, 60, 90 and 120 minutes post-glucose gavage additional blood samples were taken and immediately analyzed using a blood glucose meter (Accu-Chek Blood Glucose meter, Laval, QC).

3.2.5 Plasma and tissue collection

At study termination following an overnight fast, rats were anaesthetized with Isoflurane and a fasting cardiac blood sample was taken. Rats were then given 50%

dextrose (wt/vol) by oral gavage at a dose of 2 g/kg. At 15, 30, 60 and 90 minutes post-gavage, additional cardiac blood samples were taken according to our previous work (69). Once drawn, blood was immediately place in a cooled EDTA treated tube containing diprotinin-A (0.034 mg/ml blood; MP Biomedicals, Irvine, CA); Sigma protease inhibitor (1 mg/ml blood; Sigma Aldrich, Oakville, ON, Canada) and Roche Pefabloc (1mg/ml of blood; Roche, Mississauga, ON, Canada). After centrifugation at 1600 g for 15 min at 4^{o}C, plasma aliquots were stored in triplicate at -80^{o}C for GLP-1 (active), ghrelin (active), insulin, amylin (active), leptin, GIP (total) and PYY (total) analysis.

Following the 90-minute sample the rats were over-anesthetized followed by an aortic cut and the small intestine and colon excised, flushed and weighed. Distal segments of the duodenum, jejunum and ileum were collected and snap frozen in liquid nitrogen along with stomach, liver and proximal colon tissue. All samples were stored at –80^{o}C until analysis.

3.2.6 Plasma analysis

Ghrelin (acylated), amylin, insulin, leptin, GIP, and PYY concentrations were quantified using a Rat Gut Hormone Panel Milliplex kit (Millipore, St. Charles, MO) and Luminex instrument according to the manufacturer's specifications. The minimum sensitivity for the Milliplex kit (in pg/ml) is 1.9 (ghrelin), 20 (amylin), 1 (GIP), 28 (insulin), 27 (leptin), 16 (PYY). Active GLP-1 was quantified using an ELISA kit (Millipore, St. Charles, MO) with a minimum sensitivity of 2pM.

3.2.7 Hepatic triglyceride and non-esterified fatty acids (NEFA) assays

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

Fasting plasma samples were analyzed for NEFA using an enzymatic colorimetric assay (Wako Diagnostics, Richmond, VA).

3.2.8 RNA extraction and real-time PCR analysis

Total RNA was extracted from stomach, liver, ileum and colon tissue with TRIzol reagent (Invitrogen, Carlsbad, CA). The concentration of total RNA was quantified by Ribogreen followed by reverse transcription using the first strand cDNA synthesis kit for rt-PCR (Invitrogen) with oligo $d(T)_{15}$ as the primer. The resultant cDNA was amplified using primers synthesized by University of Calgary Core DNA Services (Calgary, AB, Canada). A BioRad iCycler (BIO-RAD, Hercules, USA) was used for the real-time PCR. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was verified as an appropriate internal control for stomach, ileum and colon tissues and β-actin for genes of interest in the liver. The $2^{-\Delta CT}$ method [$\Delta C_T = C_T$ (gene of interest) – C_T (reference gene)] was utilized for the data analysis where threshold cycle (C_T) indicates the fractional cycle number at which the amount of amplified target reaches a fixed threshold (70). Genes of interest were as follows: stomach (ghrelin); ileum and colon (proglucagon, PYY, G-coupled protein receptor (GPR41, GPR43); liver (sterol regulatory element binding protein (SREBP)–1c, fatty acid synthase (FAS) and acetyl-coenzyme A carboxylase (ACC). Primer sequences used have been published previously (6).

3.2.9 Statistical analysis

All data is expressed as mean ± standard error of the mean (SEM). A two-way analysis of variance (ANOVA) was used to evaluate differences with litter size and diet group as fixed factors followed by *Bonferroni* post hoc tests. Where no litter effect was found, normal litter and small litter rats were combined to assess diet effects. Changes in glucose and hormone levels during the OGTT and longitudinal body weight and energy intake data were analyzed with repeated measures ANOVA. Statistical significance was set at $P \leq 0.05$. Statistical analysis was performed using PASW v. 17.0 software (SPSS Inc., Chicago, IL, USA).

3.3 Results

3.3.1 Energy intake

In weeks 3 and 4, SL rats on OFS had significantly lower energy intake and in week 5 NL and SL rats weaned onto OFS consumed significantly fewer calories than C rats (Figure 3.1 A). In week 7, SL rats on control diets consumed more energy than the other groups (Figure 3.1 A). When energy intake was normalized to body weight there were no significant differences (Figure 3.1 B).

Figure 3.1 Energy intake over time

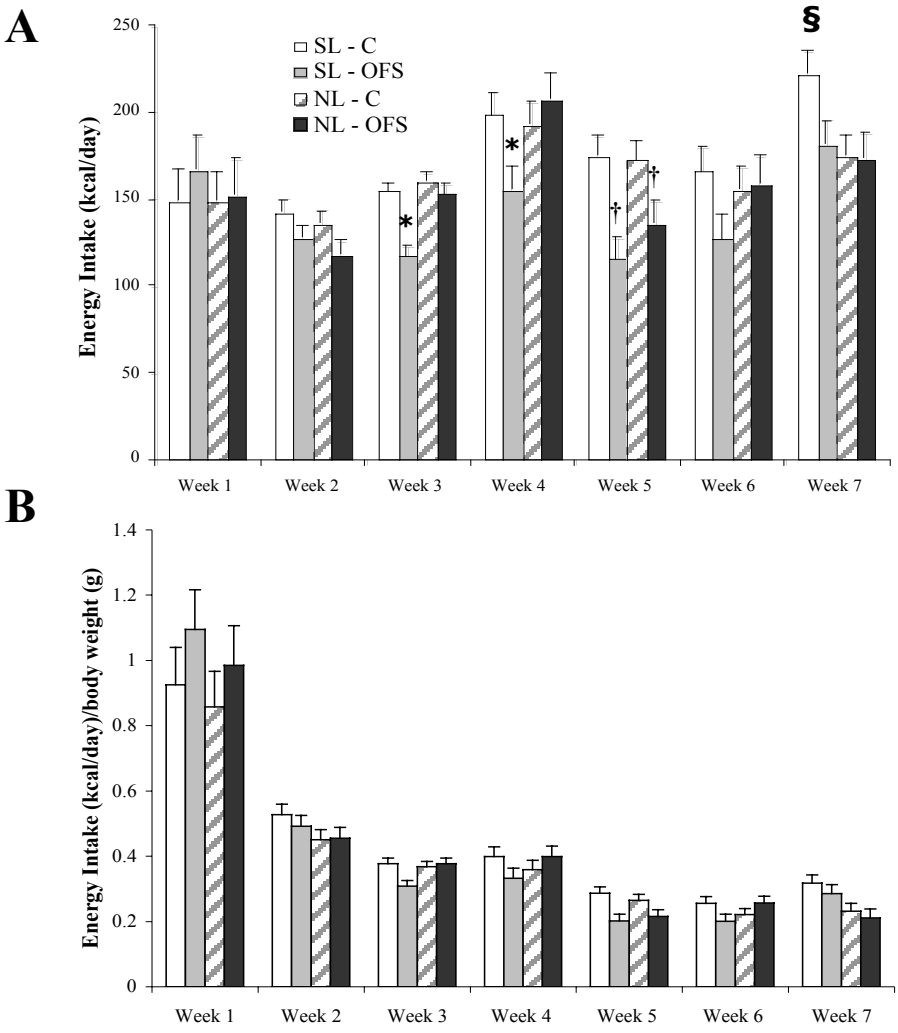

Figure 3.1 Daily energy consumption calculated from food intake data. Values represent mean ± SEM (9-10 rats/group). Panel A represents absolute energy intake. The * represents a significant interaction in the SL rat group with a decreased energy intake on OFS diet compared to C (P<0.003). The † represents a significant diet effect between C and OFS diets (P<0.003). The § represents a significant interaction in the SL rat group with increased energy intake on C diet compared to OFS diets (P<0.03). Panel B represents energy intake normalized to body weight.

3.3.2 Body composition

Similar to other work, SL rats had significantly higher body weight (55.4 ± 2.1 g) than NL rats (50.5 ± 1.1 g) at weaning (3 wk of age) (P=0.03, Figure 3.2A). Although the body weight of the OFS-fed groups were consistently lower than their C counterparts from 7 wk to 19 wk of age, these differences were statistically significant only at the end of the study (Figure 3.2B). Rats weaned onto the OFS diet had significantly lower body weight at 19 wk compared to rats weaned onto C diet (P=0.03). Contrary to the literature on NL versus SL rats in adulthood, our NL adult rats had significantly greater percent body fat (P=0.04) and fat mass (P=0.04) than SL rats (Table 3.1). However, NL-OFS rats had lower percent body fat than NL-C rats (P<0.05) whereas SL-OFS rats did not display lower percent body fat compared to SL-C rats.

Figure 3.2 Body weight over time

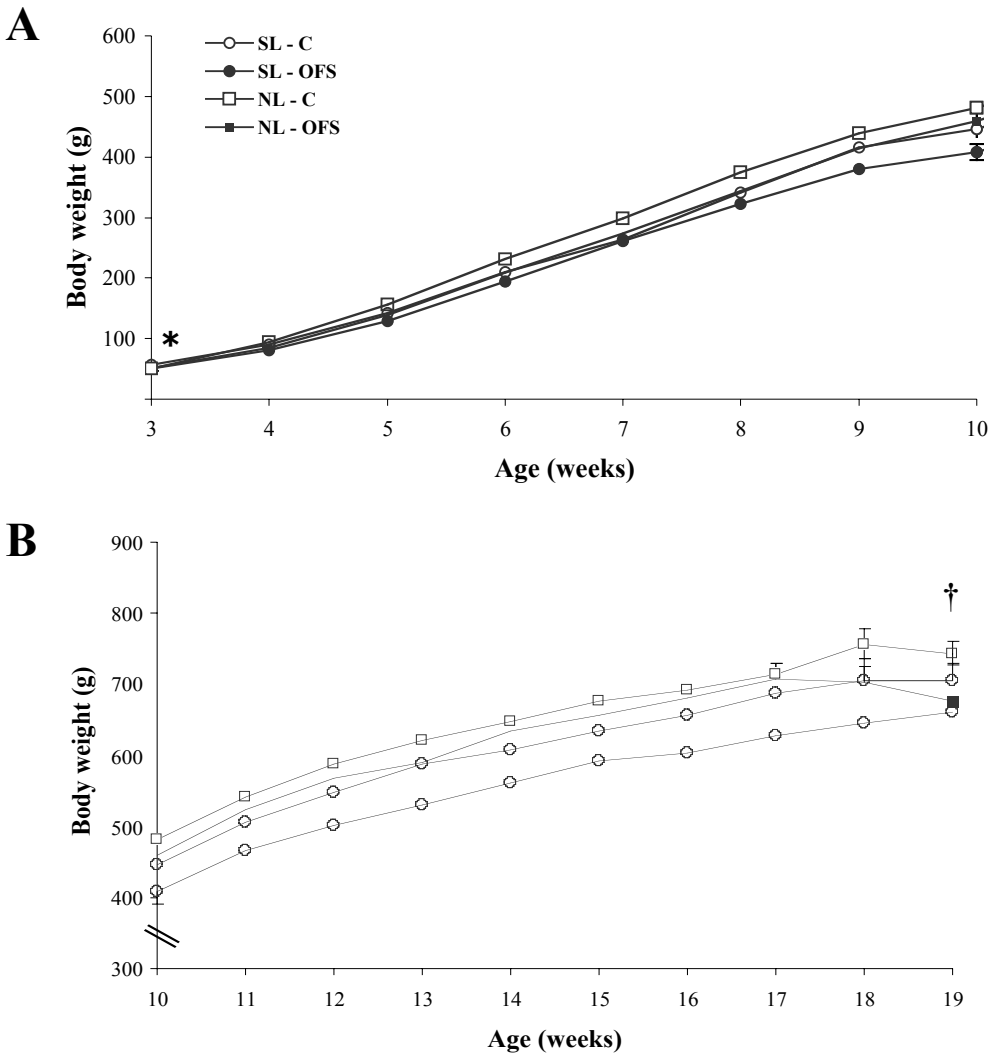

Figure 3.2 Weekly body weight in SL and NL rats fed C or OFS diet. Panel A represents body weight from weaning (3 wk) to 10 wk of age. Panel B represents body weight from 10 wk to study termination at 19 wk of age wherein the y-axis has been collapsed to better illustrate group differences. Values represent mean ± SEM (9-10 rats/group). The * in panel A represents a significant litter size effect in which SL rats were heavier than NL rats ($P<0.05$). The † in panel B represents a diet effect in which body weight was lower in OFS versus C rats at week 19 ($P<0.05$).

Table 3.1 Body Anthropometric Data

Table 3.1 Body anthropometric data analyzed by DEXA of SL and NL rats weaned onto either C or OFS diets.

	SL – C	SL – OFS	NL – C	NL – OFS	Litter Size	Diet	Interaction
					2-way ANOVA *P* values		
Weaning body weight (g)	56.7±4.9*	53.5±4.4*	50.4±1.3	51.5±2.4	0.032	0.931	0.669
Final body weight (g)	705.6±26.7	660.0±29.3†	743.4±13.5	675.9±6.2†	0.271	0.025	0.650
Body fat (%)	26.6±2.5	27.0±2.1	34.9±1.5*	28.0±2.1* §	0.036	0.135	0.045
Fat mass (g)	191.2±23.7	182.7±21.6	266.8±17.1*	196.1±18.5*	0.041	0.067	0.146
Lean mass (g)	514.4±16.3	477.4±11.7	494.1±10.7	499.0±14.2	0.960	0.242	0.130
BMD	0.18±0.002	0.18±0.003	0.18±0.001	0.18±0.002	0.950	0.922	0.087

Values are mean ± SEM (9-10 rats/group). The * represents a significant litter size effect between SL and NL rats ($P<0.05$). The † represents a significant diet effect between C and OFS diets ($P<0.05$). The § represents a significant interaction between C and OFS diets in NL rats ($P<0.05$).

3.3.3 Gastrointestinal anthropometrics

Liver weight was significantly greater in rats from NL compared to SL rats (*P*=0.01, Table 3.2). OFS was associated with significantly lower liver (*P*=0.004) and stomach (*P*=0.02) weight compared to C. There was no effect of litter size or diet on small intestine weight or length. Colon weight (*P*<0.001), cecum weight (*P*<0.001) and colon length (*P*=0.03) were all significantly greater in the rats fed OFS versus C.

Table 3.2 Gastrointestinal Anthropometrics

Table 3.2 Gastrointestinal anthropometrics collected at sacrifice in SL and NL rats weaned onto either C or OFS diets.

	SL – C	SL – OFS	NL – C	NL – OFS	Litter Size	Diet	Interaction
					2-way ANOVA *P* values		
Liver weight (g)	22.2±0.7	19.0±1.2*	25.4±0.9†	21.7±1.6*†	0.011	0.004	0.827
Stomach weight (g)	3.0±0.1	2.4±0.1*	2.9±0.2	2.7±0.2*	0.392	0.02	0.218
Small intestine weight (g)	8.9±0.4	8.8±0.3	9.5±0.1	8.9±0.3	0.269	0.254	0.524
Small intestine length (cm)	142.1±3.8	145.9±3.3	147.6±1.6	145.0±2.8	0.463	0.854	0.312
Cecum weight (g)	1.2±0.09	2.9±0.22*	1.1±0.05	2.8±0.29*	0.636	0.001	0.879
Colon weight (g)	1.69±0.08	2.10±0.11*	1.60±0.05	2.03±0.16*	0.529	0.001	0.846
Colon length (cm)	21.9±1.0	23.8±1.0*	21.8±1.3	25.0±1.3*	0.641	0.033	0.558

Values are mean ± SEM (9-10 rats/group). The * represents a significant diet effect between C and OFS diets ($P<0.05$). The † represents a significant litter size effect between SL and NL rats ($P<0.05$).

3.3.4 Plasma glucose, insulin, amylin and leptin responses

In NL rats, those fed OFS had significantly lower blood glucose levels at every time point during the OGTT than those fed C diet ($P=0.009$, Figure 3.3A). OFS consumption significantly reduced total AUC for glucose in NL but not SL rats ($P=0.05$, Figure 3.3B). Total AUC glucose was significantly correlated with body fat (%) ($r^2=0.56$, $P<0.001$, Figure B.1). There was no difference in serial values during the OGTT or total AUC for insulin between litter sizes or diets (Figures 3.3 C and D). It should be noted however that rats raised in NL had total AUC insulin levels that were approximately 10 ng/ml lower when fed OFS compared to C whereas there was no noticeable change in the insulin levels of rats raised in SL between OFS and C diets. Plasma levels of amylin did not differ between litters or diets (Figures 3.3 E and F). Leptin total AUC tended to be lower in rats fed OFS ($P=0.06$, Figure 3.3 H) compared to rats fed C and there was a significant correlation between leptin and body fat (%) for fasting and total AUC levels ($r^2=0.51$, $r^2=0.72$, $P<0.003$, $P<0.001$ respectively, Table 3.3, Figure B.1 and B.2).

Figure 3.3 Plasma glucose, insulin, amylin and leptin responses

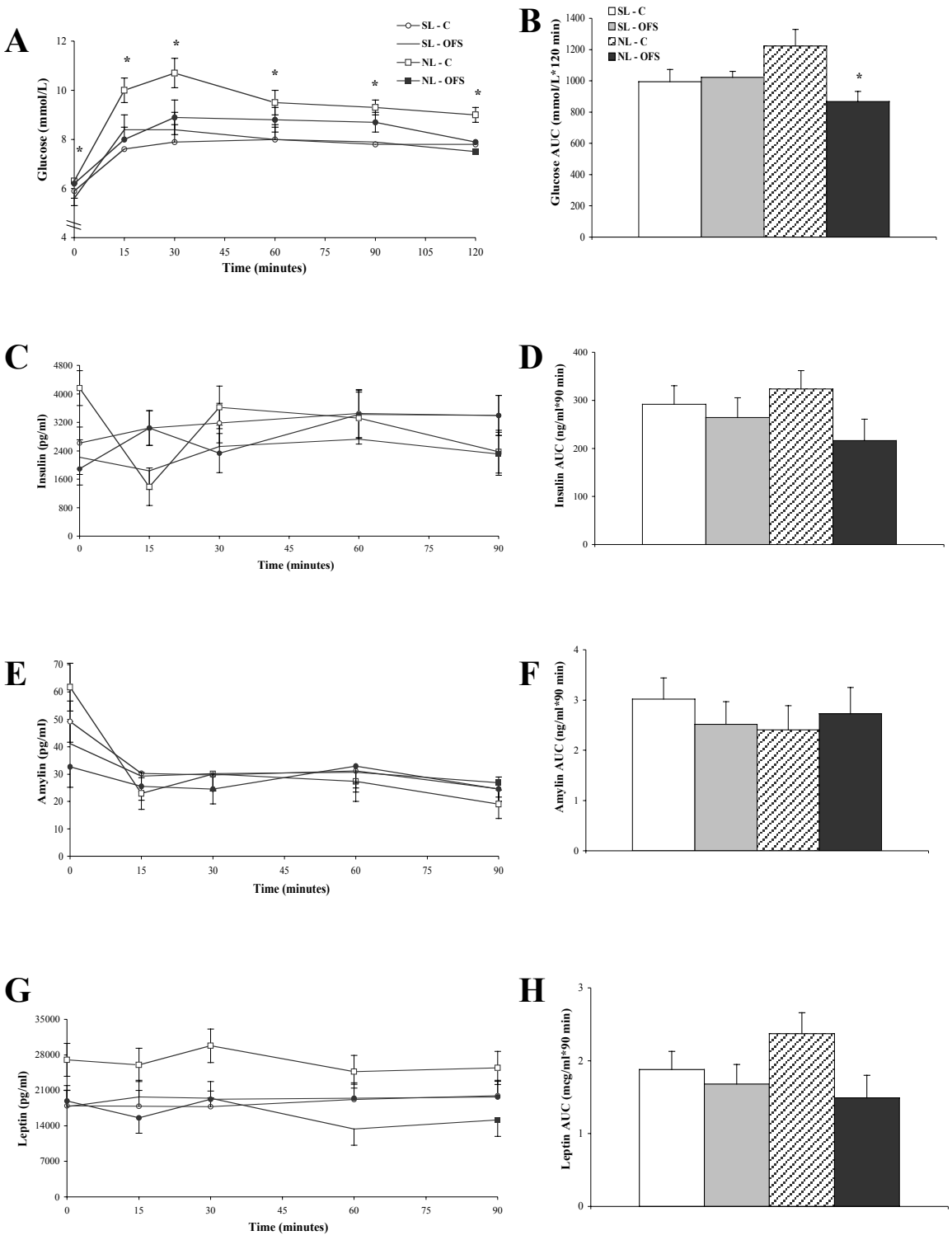

Figure 3.3 Blood glucose and plasma insulin, amylin, and leptin responses during an OGTT. The left column represents serial values at individual time points and the right column represents total AUC (units adjusted for graphical presentation). Values represent mean ± SEM (9-10 rats/group). The * in panels A and B represents a significant interaction between C and OFS diets in NL rats ($P<0.05$).

3.3.5 Plasma GLP-1, PYY, GIP and ghrelin responses

At 30 minutes post-glucose load, GLP-1 levels in rats fed OFS were significantly higher than rats fed C ($P=0.02$, Figure 3.4A). Total AUC for GLP-1 was significantly higher in OFS rats than C rats but did not differ between litter sizes ($P=0.02$, Figure 3.4B). Rats fed OFS had significantly higher PYY concentrations at every time point during the OGTT (Figure 3.4C) and expressed as total AUC (Figure 3.4D) ($P<0.001$). There was a trend for rats raised in SL to have higher PYY concentrations compared to rats raised in NL ($P=0.058$). Total AUC for GIP was significantly lower in rats fed OFS compared to rats fed C ($P<0.001$, Figure 3.4F). Plasma levels of ghrelin did not differ between litters or diets (Figures 3.4G and H) but fasting levels of ghrelin were significantly negatively correlated with body fat (%) ($r^2=-0.34$, $P=0.05$, Table 3.3, Figure B.2).

Figure 3.4 Plasma GLP-1, PYY, GIP and ghrelin responses

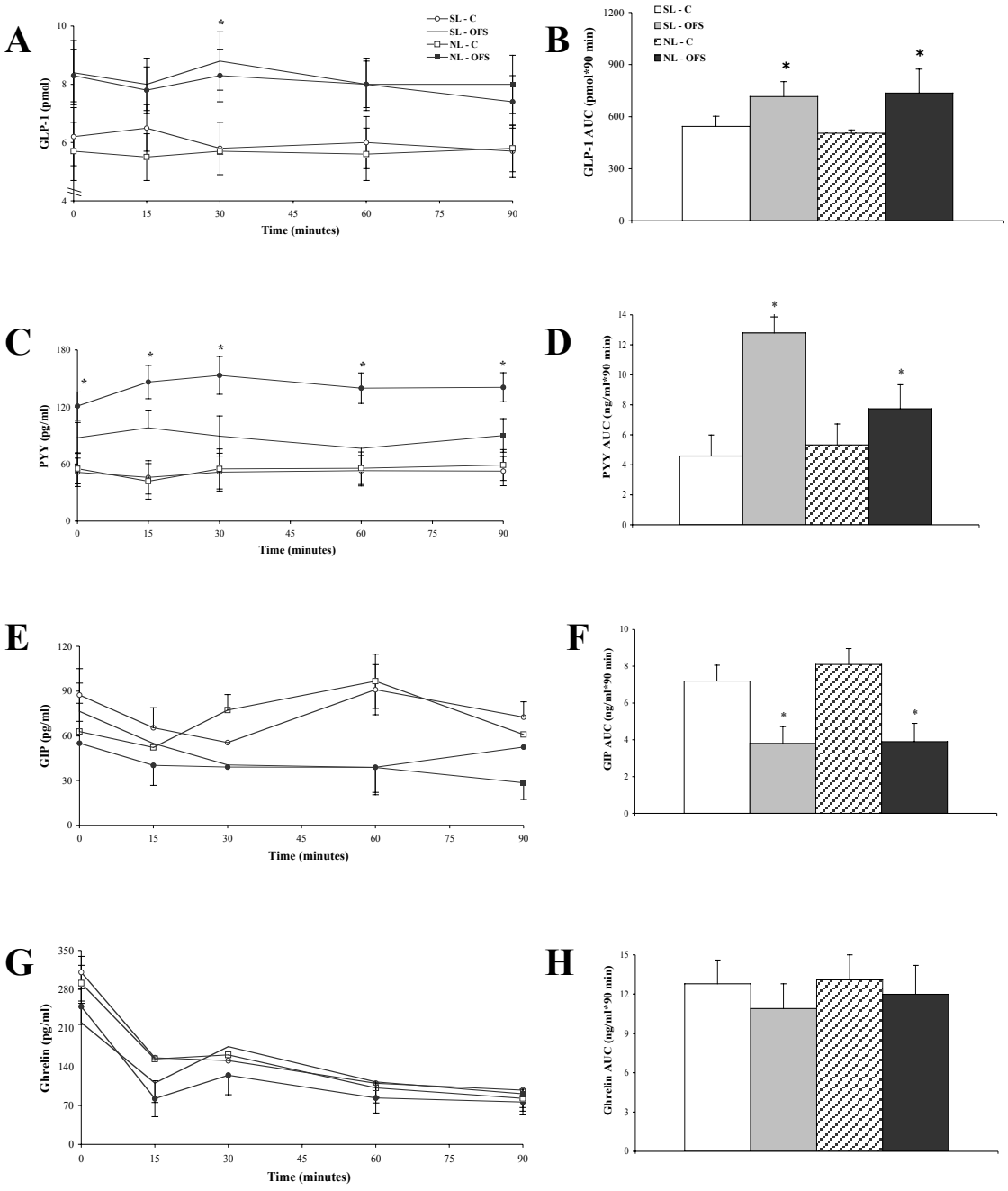

Figure 3.4 Plasma GLP-1, PYY, GIP and ghrelin responses during an OGTT. The left column represents serial values at individual time points and the right column represents total AUC (units adjusted for graphical presentation). Values represent mean ± SEM (9-10 rats/group). The * represents a significant diet effect between C and OFS diets ($P<0.05$).

Table 3.3 Correlation of body fat (%) with plasma glucose, insulin and satiety hormones

Table 3.3 Correlation of body fat (%) as measured by DEXA with plasma metabolites at both fasting and total AUC for SL and NL rats weaned onto C or OFS diets.

	Fasting		**AUC**	
	Correlation	***P*-value**	**Correlation**	***P*-value**
Glucose	0.25	0.16	0.56	0.001**
Insulin	0.04	0.86	0.26	0.17
Amylin	-0.02	0.93	-0.09	0.64
Leptin	0.51	0.003*	0.72	0.001**
GLP-1	-0.17	0.36	-0.02	0.93
PYY	-0.15	0.41	-0.18	0.34
GIP	-0.34	0.07	0.33	0.08
Ghrelin	-0.34	0.05*	-0.12	0.53

* represents a significant correlation between body fat (%) and fasting plasma metabolite.

** represents a significant correlation between body fat (%) and total AUC plasma metabolite.

3.3.6 Hepatic triglyceride and plasma NEFA levels

Triglyceride content of the liver did not differ between litters but tended to be lower in NL rats fed OFS (P=0.10, Figure 3.5). Similarly, there was no significant litter size effect for plasma NEFA concentrations and the approximate 42% decrease in NEFA seen in OFS-fed rats did not differ significantly from C (P>0.05).

Figure 3.5 Liver triglyceride content and circulating NEFA levels

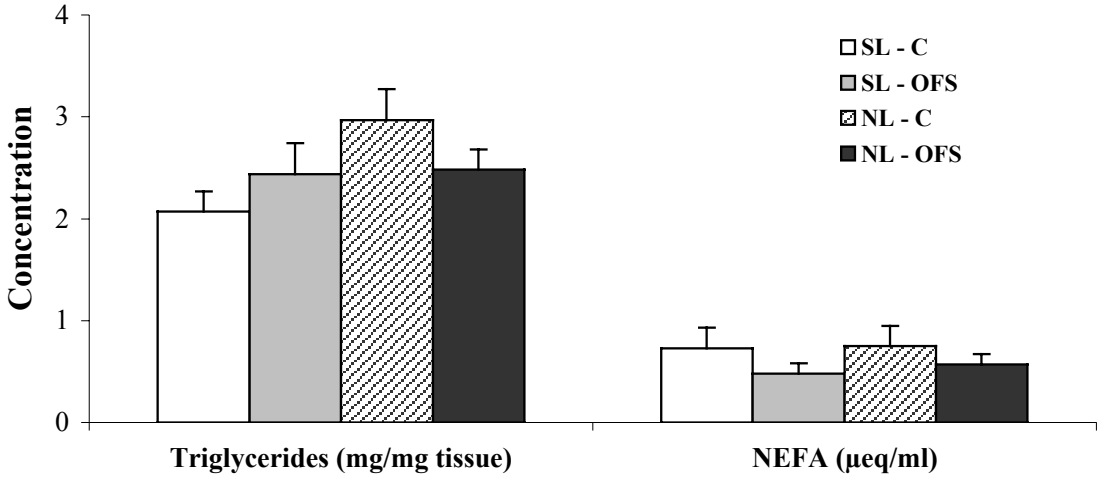

Figure 3.5 Hepatic triglyceride content (mg/mg tissue) and plasma non-esterified fatty acids (µeq/ml) in SL and NL rats fed C or OFS diet. Values represent mean ± SEM (9-10 rats/group).

3.3.7 Gene expression

In the colon, proglucagon mRNA levels were significantly different in rats raised in SL versus NL (P=0.04) and were nearly 2-fold higher in SL-OFS rats compared to SL-C (Figure 3.6A). In contrast, NL rats had higher proglucagon mRNA levels in the ileum compared to SL rats (P=0.057). There was no difference in the expression of PYY in either the ileum or colon for both SL and NL rats on OFS or C. mRNA levels of the SCFA receptor, GPR43, were significantly higher with OFS versus C (P=0.05, Figure 3.6B).

In the liver, FAS mRNA levels were significantly higher in rats raised in NL compared to SL (P=0.007, Figure 3.7). There was no effect of OFS on FAS mRNA levels and SREBP–1c expression was not affected by litter size or diet. There was a 57% increase in ACC in rats raised in NL compared to SL that was not significant (P=0.08).

Figure 3.6 Small intestine and colon gene expression

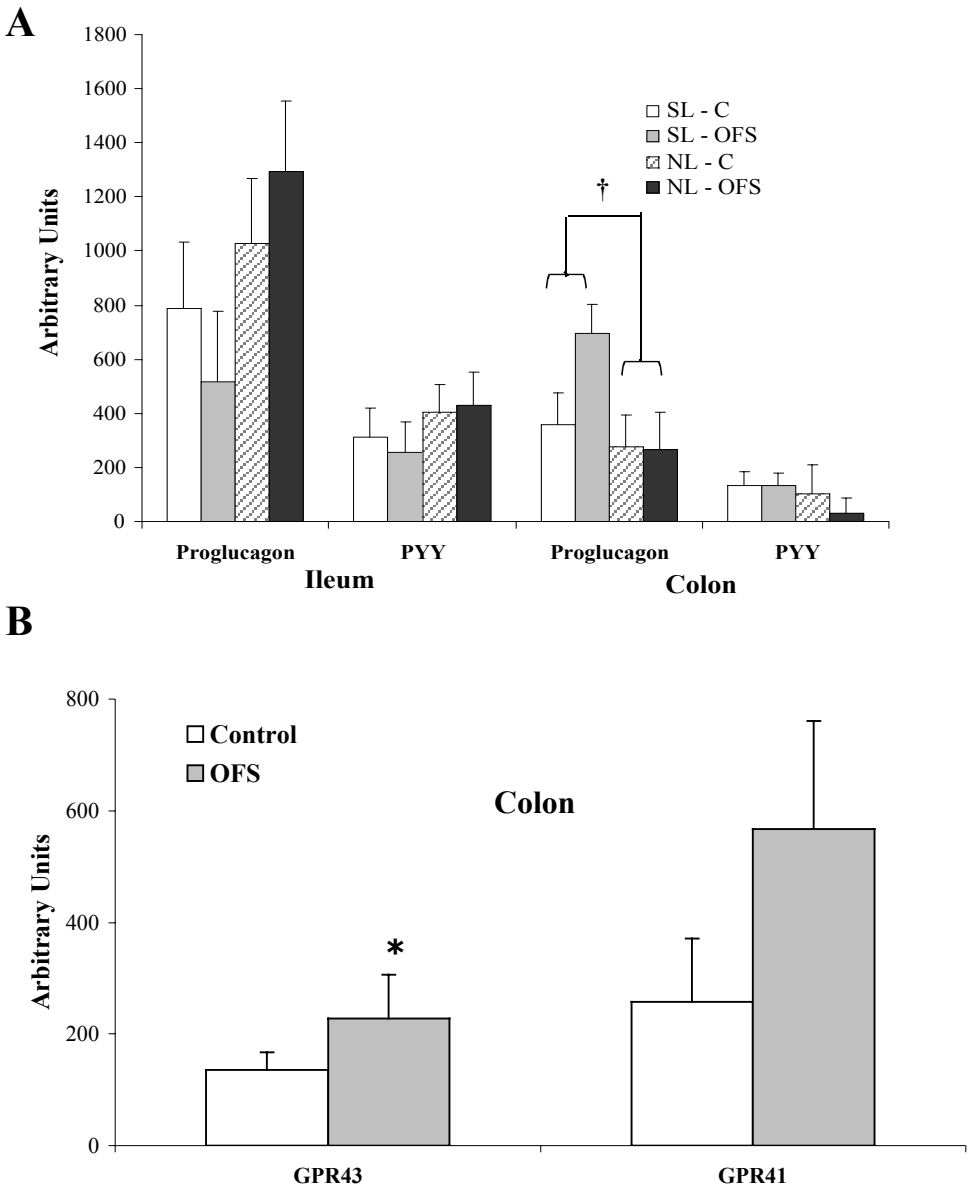

Figure 3.6 Expression of proglucagon, PYY and GPR41 and 43 in the intestine of SL and NL rats fed C or OFS diet. Values represent mean ± SEM (9-10 rats/group). The † in panel A represents a significant litter size effect between SL and NL rats (P<0.05). The * in panel B represents a significant diet effect in the GPR43 expression in the colon between C and OFS diets (P<0.05).

Figure 3.7 Hepatic gene expression

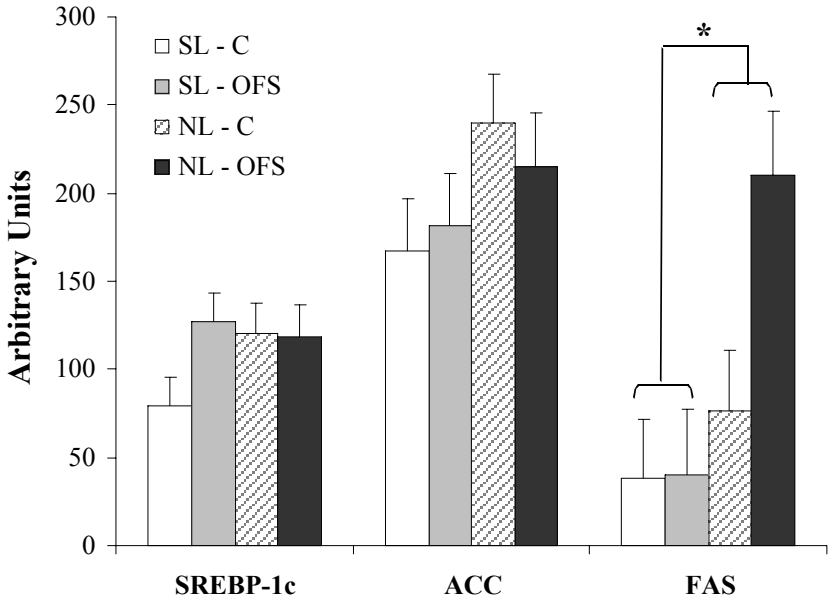

Figure 3.7 Expression of FAS, ACC, and SREBP-1c in the liver of SL and NL rats fed C or OFS diet. Values represent mean ± SEM (9-10 rats/group). The * represents a significant litter size effect between SL and NL rats (P<0.05).

3.4 Discussion

The purpose of this study was to assess the potential for prebiotic fiber supplementation to reverse programmed obesity susceptibility in young rats. Raising rats in litters of three pups allowed the pups greater access to nutrition throughout the suckling period (24). Overnutrition during this critical stage of post-natal growth often results in adverse changes in metabolism that can lead to obesity and hyperglycemia in these animals (11, 21, 26). This study was designed to evaluate satiety hormone secretion

and glycemic control in oversuckled rats weaned onto a control diet or an OFS-enriched diet that has previously been shown to reduce body fat and postprandial glycemia in adult rats (6, 41, 71). Although both SL and NL rats exhibited a favourable response to OFS, the SL rats appeared to have a blunted ability to fully respond to the hypoglycemic and adiposity-reducing effects of OFS.

3.4.1 Body composition and energy consumption

As expected, pups raised in SL where heavier at weaning compared to pups raised in larger, normal sized litters (65). This increase in body weight compared to NL pups did not persist into adulthood which is consistent with some (24, 72, 73) but not all work in the area (20-22, 74-76). The range of litter sizes (2-8 pups/litter) that are classified as oversuckled rats in the literature limits our ability to generalize our findings related to body composition to other studies (19, 22, 64, 77). Although the NL-C rats had a higher percent body fat and greater fat mass at sacrifice than the SL-C rats, NL-OFS rats experienced a reduction in fat mass of approximately 75g compared C, whereas SL-OFS rats only experienced a decrease of ~10g of body fat compared to C. The differences in energy consumption between the litters may have accounted for the differences in fat mass. However when energy intake was normalized to body weight these differences were no longer apparent. This indicates that all of the rats consumed the same amount of energy to sustain their body weight. Additionally, each group of rats consumed the same amount of fiber for their body weight demonstrating that any changes in body composition may be due to changes in metabolism and not primarily due to differences in fiber consumption.

This blunted response to the fat-reducing effects of OFS highlights the need to further examine adipose tissue metabolism and its sensitivity to the actions of OFS. Changes in adipose tissue size as well as cell number and metabolism, such as increased cholesterol synthesis, have been previously described for oversuckled rodent models (11, 21). In a similar study, Wiedmer et al. showed that the slightly higher adiposity in male Wistar rats raised in litters of 2 pups was not due to lower energy expenditure and therefore suggested that another cellular pathway must play a role in interrupting normal energy homeostasis in these animals (73). Further examination of the exact changes in energy utilization related to prebiotic fiber consumption within the visceral white adipose tissue is warranted.

3.4.2 Satiety hormone response

The increased secretion of GLP-1 observed in the OFS rats is a well-documented outcome of prebiotic fiber consumption (41, 78). Cani et al. demonstrated that male Wistar rats fed an OFS-enriched diet exhibited a doubling in the concentration of GLP-1 in the hepatic portal vein as well as upregulation in proglucagon mRNA expression in the proximal colon compared to standard chow diet (41). In the current study a distinctly different transcriptional response was seen in that OFS increased proglucagon mRNA levels in the colon of SL but not NL rats. At this point it is unclear which mechanism(s) related to OFS feeding in oversuckled rats may have stimulated these enteroendocrine cell changes. It has been suggested that SCFA products from the bacterial fermentation of non-digestible carbohydrates such as OFS may be responsible for modulating gene expression in the colon (79). Although the increased expression of proglucagon in the SL rats may have contributed to the overall significantly higher secretion of GLP-1 in the

plasma of OFS rats, the SL rats did not have a robust increase in the secretion of GLP-1 compared to NL rats. Whether defects in the secretory machinery of the L-cells in SL rats account for the blunted GLP-1 response to increased proglucagon mRNA levels warrants further investigation.

The other major incretin, GIP, was reduced in response to OFS. These findings confirm our recent observation of reduced GIP levels in obese rats treated with a combination of OFS and the insulin sensitizing drug metformin. In general, postprandial GIP secretion has been shown to be increased in obese patients compared with age matched healthy individuals (80). In rodent models, inhibiting GIP receptor signalling can prevent many of the metabolic abnormalities associated with dietary-induced obesity-diabetes (81). Given the suggestion that GIP is involved in mediating the promotion of triglyceride storage, it is plausible that some of the effects of OFS on lipid metabolism are mediated via reduced circulating GIP (80).

Another potential contributor to the decreased weight gain in OFS rats is the significantly higher level of circulating PYY. Although there was no detectible change in gene expression for PYY in the ileum or colon there was a trend for SL-OFS rats to have the greatest magnitude of PYY secretion. There is evidence that bacterial fermentation of prebiotic in the large bowel produces SCFA that may directly influence PYY secretion (79). In addition to being absorbed and used as an energy source, SCFA have been found to be ligands for certain GPR including GPR43 and GPR41 that have been linked with the promotion of PYY secretion (79, 82). Indeed, we found an increased expression of GPR41 and 43 with OFS, although only GPR43 was significant. Prebiotic fiber supplementation in rodent models has been shown to increase *Bifidobacterium* and

Lactobacilli species and contribute to improved insulin sensitivity, increased satiety hormone secretion and decreased body fat (83). It is possible that changes in gut microbiota are at least in part responsible for changes in GPR and satiety hormone mRNA levels.

It is noteworthy that no significant decrease in energy intake over time was observed in the rats and this may be linked to an alteration in the sensitivity of the Y2 receptor for PYY in the brain (36). Changes in the sensitivity of orexigenic and anorexigenic hormones in the paraventricular hypothalamus (PVH) of oversuckled rats has been previously demonstrated (35). In this case, although OFS may have stimulated PYY secretion the full benefits of this peptide may have been hampered by an altered PYY sensitivity in the central nervous system. Alterations in the central nervous system induced by post-natal oversuckling may be particularly difficult to override.

3.4.3 Plasma glucose and insulin responses

OFS has been shown to lower postprandial glycemia in normal and streptozotocin-induced diabetic rats (59). We found similar results with NL-OFS rats showing significantly lower postprandial glucose levels compared to NL-C rats. However, lower plasma glucose was not observed in SL-OFS rats compared to SL-C rats. A strong inverse relationship between GLP-1 secretion and postprandial glycemia has been shown, however, this association appears blunted in the SL rats (36). A similar response to insulin was also found with NL rats having a difference of 10.7 ng/ml in total AUC in OFS fed rats compared to control whereas SL rats only exhibited a decrease of 2.7 ng/ml in insulin levels on OFS.

Mechanisms influencing central and peripheral-mediated insulin sensitivity have been studied in response to dietary programming (11, 20, 72, 76). Alterations in insulin signalling at the arcuate nucleus in the hypothalamus as well as morphological changes to pancreatic islet cells have been recorded in both growth-restricted and oversuckled rat models. There is evidence that neonatal oversuckling results in the decreased anorexigenic signalling capacity of insulin leading to a reduction in the satiating effect of this hormone and hyperinsulinaemia (76). From this current study it appears that post-natal oversuckling may have additionally altered the incretin-insulin signalling pathway leading to impaired response of insulin to the postprandial effects of GLP-1.

3.4.4 Lipid metabolism

OFS feeding has been shown to be a powerful mediator of liver triglyceride levels and circulating free fatty acid concentration; decreasing levels in both humans and rodents (38). Although levels did not reach significance, rats fed OFS had both lower hepatic triglyceride levels and decreased circulating free fatty acid concentrations compared to C diet. We have recently demonstrated that OFS supplementation increases the activation of AMP-activated protein kinase (AMPK) in the liver. Activation of the AMPK pathway is tightly linked to reductions in plasma glucose and triglycerides (84).

Although OFS feeding tended to decrease the total triglyceride content in the liver the inverse was observed for the mRNA expression of lipogenic genes in the liver. A paradoxical relationship emerged between feeding OFS and the expression of FAS, especially in the NL rats. It would seem that although circulating levels of both insulin and glucose were reduced in NL rats on OFS, there was a significant upregulation of FAS and ACC to a lesser extent, in the liver. Since insulin is thought to be a promoter of FAS

expression and OFS consumption has been linked to decreased FAS expression this result is surprising (78, 85). It should be noted that neither SREBP-1c nor ACC, upstream regulators of FAS, were changed to any significant extent. The increased percent body fat in the NL rats may be attributed to the increased FAS expression but further research is needed to understand how OFS influences lipogenesis in the liver (85).

3.5 Conclusion

In conclusion we demonstrated that OFS was able to positively influence certain aspects of metabolism in oversuckled rats. Both GLP-1 and PYY were increased with OFS feeding in SL rats and triglyceride content in the liver was decreased. However, the ability of OFS to reduce body fat and glycemia was blunted in SL rats suggesting that oversuckling from birth to weaning had lasting deleterious effects that could not be overcome by fiber supplementation alone. Introduction of a prebiotic-enriched diet immediately following the oversuckling period proved to be a plausible approach to partly reversing programmed obesity risk. Further study of specific changes in metabolic pathways controlling energy balance in oversuckled rats may help in the development of dietary and pharmacological targets for reversing metabolic dysfunction.

Chapter Four: Rats raised in large versus small litters respond differently to the satiating effects of oligofructose when added to a high fat/sucrose diet[2]

4.1 Introduction

Obesity has reached global epidemic proportions and is becoming the fastest growing contributor of metabolic diseases in many Western countries (4). Adding to the total increase in obesity is the significant rise in childhood obesity (2). It has been suggested that there is a relationship between excessive adiposity in childhood and an increased tendency for overweight and obesity in adulthood. Related to an accelerated growth pattern in early life is an increased risk of developing type 2 diabetes, cardiovascular disease and certain cancers (3). Addressing the metabolic factors and genetic predisposition that influence excessive childhood adiposity is one strategy aimed at reducing the development of obesity in adulthood (11).

Rodent models have been valuable in elucidating the metabolic changes in offspring exposed to adverse nutrition *in utero* (17, 21, 30, 86). Specifically, rat dams exposed to high fat or high carbohydrate diets during pregnancy and lactation result in offspring that can be obese, hyperglycaemic and hypertensive (24, 77). These models have demonstrated the potential for *in utero* malnutrition to elicit epigenetic changes in the offspring (30). There is a lesser amount of work that has specifically addressed postnatal dietary changes that also have the potential to impart significant long-term

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[2] This work was presented in part at The Obesity Society Annual Scientific Meeting, San Diego, Oct. 8-12, 2010. Reid DT, Reimer RA (2010) Supplementing a high fat/sucrose diet with prebiotic fiber (oligofructose) differentially affects glucose metabolism and appetite in rats raised in small versus large litters. Obesity 18:S73

metabolic consequences, a phenomenon that has been termed perinatal programming (15, 22, 24, 73).

The oversuckled rat model, achieved by reducing litter size at birth, is one example of overnutrition during early postnatal development that leads to pups that are overweight and demonstrate deleterious changes in metabolic function in adulthood (24). Using this model we have previously demonstrated that oversuckled male rats weaned onto prebiotic fiber-enriched diets had a limited capacity to respond to the adiposity-reducing effects of OFS. The rats raised in small litters (SL, 3 pups/litter) were also hyperglycaemic and hyperinsulinaemic compared to the rats raised in normal litters (NL, 12 pups/litter) despite having a lower body weight and fat mass. Although the SL rats responded to OFS feeding by increasing the secretion of the anorexigenic hormone GLP-1, the magnitude of the response was less than in NL rats.

There is evidence that the background diet to which OFS is supplemented can influence the response of the animal to the fiber (71). Given the differences we observed in oversuckled rats weaned onto normal energy density diets that contained OFS, our goal in this work was to determine the effect of a high fat/high sucrose background diet on oversuckled rats and the potential for metabolic change in these rats when their diet was enriched with OFS. There are mixed reports in the literature showing both detrimental and protective effects of feeding oversuckled rats a high energy diet into adulthood. Faust et al. demonstrated that the metabolic dysfunction passed on by oversuckling was exacerbated with high fat feeding into adulthood (86). On the contrary, Khan et al. demonstrated that high fat feeding in male offspring from dams fed a high fat diet during pregnancy and lactation had lower circulating glucose and less endothelial dysfunction

compared to offspring weaned onto standard chow (87). To further understand the long-term metabolic changes produced by oversuckling, the objective of this study was to examine whether OFS supplementation could mitigate the detrimental effects of oversuckling in rats weaned onto high fat/high sucrose diet. It was hypothesized that rats fed OFS would have reduced body fat gain and display greater satiety hormone response compared to animals fed the control high fat/high sucrose diet.

4.2 Methods

4.2.1 Animals and housing

The study protocol was approved by The University of Calgary Animal Care Committee and conformed to the *Guide for the Care and Use of Laboratory Animals*. Twenty, first-time pregnant Sprague-Dawley female rats were obtained from Charles River (St. Constant, QC) and kept on a 12-hour light-dark cycle in a temperature and humidity controlled room in the University of Calgary Animal Resource Centre. Rats were approximately one week pregnant at the time of arrival in our facility.

One day after birth, litters were randomized to become small litters (SL, culled to 3 male pups) or normal litters (NL, culled to 12 pups including both males and females). Decreasing litter size to 3-4 pups at birth and throughout the suckling period is associated with overnourishment and results in rats that are obese, hyperglycaemic and insulin resistant compared to rats raised in normal litters (10-12 pups in size) (24). At weaning, male rats were randomized into the experimental diet groups and housed in pairs (9-10 rats per diet group). Each SL rat group came from 6-7 different litters and each NL rat group was combined from 4-5 different litters.

4.2.2 Diets

Custom high fat/high sucrose (HE) diet was obtained from Dyets Inc (Bethlehem, PA). Oligofructose (OFS) (Raftilose P95) was provided by Quadra Chemicals (Vaudreuil-Dorion, PQ, Canada). All diets met the nutritional requirements of rats during growth (up to 10 wk of age) or maintenance of adult rats (10 wk onwards). At weaning, control (high energy density, HE) rats received high fat/high sucrose diet (4.6 kcal/g) for the remainder of the study. The HE diet provided 40% of energy from fat and 45% from sucrose and was composed of (g/100g): cornstarch (5); casein (14), sucrose (51), soybean oil (10), lard (10), Alphacel (5), AIN-93M mineral mix (3.5), AIN-93 vitamin mix (1), L-cystine (0.3), and choline bitartrate (0.25). OFS-supplemented diet contained 10% fiber by weight and had an energy density of 4.3 kcal/g. The OFS diet was prepared by mixing 90 g of high fat/high sucrose diet with 10 g of OFS. Food and water was provided *ad libitum* and food intake measurements were made throughout the study. Food intake was measured by weighing the difference in feed cups from one day to the next accounting for any spillage. Food intake was recorded over a week for a total of 7 independent weeks throughout the study (approximately every 2 weeks).

4.2.3 Body weight and composition measurements

Body weight was recorded using an electronic scale on the same day of every week for the duration of the study. One day before sacrifice, under light anaesthetic (Isoflurane) lean mass, fat mass and BMD was measured via DEXA using software for small animals (Hologic QDR 4500, Hologic, Inc., Bedford, MA).

4.2.4 Oral glucose tolerance test

One week prior to sacrifice an OGTT was performed to assess the blood glucose response. Following an overnight fast, blood was sampled via tail nick followed by an oral glucose gavage (2 g/kg). At 15, 30, 60, 90 and 120 minutes post-glucose gavage, additional blood samples were taken and immediately analyzed using a blood glucose meter (Accu-Chek Blood Glucose meter, Laval, QC).

4.2.5 Plasma and tissue collection

On the day of sacrifice, after an overnight fast and under anesthesia (Isoflurane), a second OGTT to assess satiety hormone response was performed using an oral glucose gavage (2 g/kg). A fasted cardiac blood sample was collected prior to the oral glucose load and then at time 15, 30, 60, and 90 minutes post-glucose gavage (69). Once drawn, blood was immediately place in a cooled EDTA treated tube containing: diprotinin-A (0.034 mg/ml blood; MP Biomedicals, Irvine, CA); Sigma protease inhibitor (1 mg/ml blood; Sigma Aldrich, Oakville, ON, Canada) and Roche Pefabloc (1mg/ml of blood; Roche, Mississauga, ON, Canada). After centrifugation at 1600 g for 15 min at 4ºC, plasma aliquots were stored in triplicate at -80^{o}C for GLP-1 (active), ghrelin (active), insulin, amylin (active), leptin, GIP (total) and PYY (total) analysis.

Following the 90-minute sample the rats were over-anesthetized followed by an aortic cut and the small intestine and colon excised, flushed and weighed. Distal segments of the duodenum, jejunum and ileum were collected and snap frozen in liquid nitrogen along with stomach, liver and proximal colon tissue. All samples were stored at –80^{o}C until analysis.

4.2.6 Plasma analysis

Ghrelin (acylated), amylin, insulin, leptin, GIP, and PYY concentrations were quantified using a Rat Gut Hormone Panel Milliplex kit (Millipore, St. Charles, MO) and Luminex instrument according to the manufacturer's specifications. The minimum sensitivity for the Milliplex kit (in pg/ml) is 1.9 (ghrelin), 20 (amylin), 1 (GIP), 28 (insulin), 27 (leptin), 16 (PYY). Active GLP-1 was quantified using an ELISA kit from LINCO research (Millipore, St. Charles, MO) and has a minimum sensitivity of 2pM.

4.2.7 Hepatic triglyceride and plasma NEFA assays

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

Fasting plasma samples were analyzed for NEFA using an enzymatic colorimetric assay (Wako Diagnostics, Richmond, VA).

4.2.7 RNA extraction and real-time PCR analysis

Total RNA was extracted from stomach, liver, ileum and colon tissue with TRIzol reagent (Invitrogen, Carlsbad, CA) following the manufacturer's directions. The concentration of total RNA was quantified by Ribogreen followed by reverse transcription using the first strand cDNA synthesis kit for RT-PCR (Invitrogen) with oligo $d(T)_{15}$ as the primer. The resultant cDNA was amplified using primers synthesized by University of Calgary Core DNA Services (Calgary, AB, Canada). A BioRad iCycler (BIO-RAD, Hercules, USA) was used for the real-time PCR reactions. GAPDH primers were verified as appropriate internal controls for stomach, ileum and colon tissues and β-actin primers for genes of interest in the liver. The $2^{-\Delta CT}$ method [$\Delta C_T = C_T$ (gene of

interest) – C_T (reference gene)] was utilized for the data analysis where threshold cycle (C_T) indicates the fractional cycle number at which the amount of amplified target reaches a fixed threshold (70). Genes of interest were as follows: stomach (ghrelin); ileum and colon (proglucagon and PYY); liver (SREBP-1c, FAS and ACC). Our previously published primer sequences were utilized (6).

4.2.8 Statistical analysis

All data is expressed as mean ± SEM. A two-way ANOVA was used to evaluate differences with litter size and diet group as fixed factors followed by B*onferroni* post hoc tests. Where no litter effect was found, normal litter and small litter rats were combined. Statistical significance was set at $P \leq 0.05$. Changes in glucose and hormone levels during the OGTT and longitudinal body weight and energy intake data were analyzed with repeated measures ANOVA. Statistical analysis was performed using PASW v. 17.0 software (SPSS Inc., Chicago, IL, USA).

4.3 Results

4.3.1 Energy intake

In weeks 1, 3 and 7, SL rats fed OFS consumed significantly more calories than SL rats fed HE ($P< 0.05$, Figure 4.1A). In week 4, NL rats fed HE had significantly higher energy intake than NL rats fed OFS ($P<0.001$) and both SL and NL rats of OFS consumed significantly less calories than SL and NL rats on HE ($P< 0.008$). Rats fed OFS consumed significantly more energy than rats fed HE when normalized for body weight ($P<0.002$, Figure 4.1B). SL-OFS rats tended to consume more calories per each gram of body weight than SL-HE rats or NL rats fed HE or OFS ($P=0.06$).

Figure 4.1 Energy intake over time

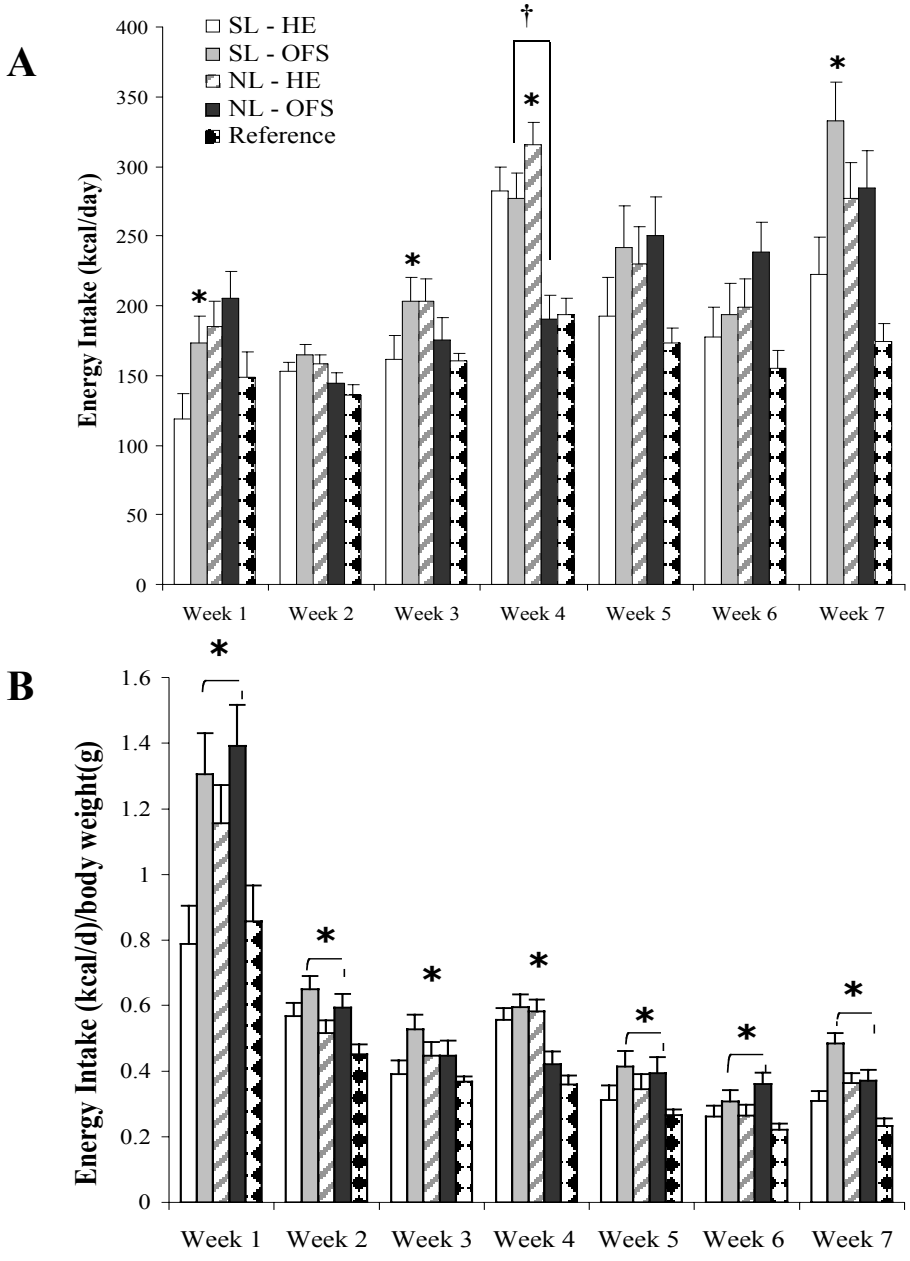


Figure 4.1 Daily energy consumption calculated from food intake data. Values represent mean ± SEM (9-10 rats/group). The * in panel A represents a significant interaction between HE and OFS diets in the SL rats and the † indicates a significant diet effect between HE and OFS diets (P<0.05). In panel B, values were calculated by dividing daily

energy intake by body weight for each individual rat. The * represents significant diet effect in rats fed OFS versus HE diets (P<0.002). Reference group consists of male rats raised in litters of 12 pups weaned onto AIN-93G (3.8 kcal/g) and at 10 weeks of age were switched onto AIN-93M (3.6 kcal/g). Reference group not included in statistical analysis.

4.3.2 Body composition

There was no difference in average body weight between the SL and NL rats measured in the first week after birth (15.3 ± 0.4 g and 15.0 ± 0.2 g in SL and NL respectively). At weaning (3 wk), SL rats had significantly higher body weight (55.7 ± 3.6 g) than NL rats (50.4 ± 1.8 g) (P <0.05, Figure 4.2). Week to week over time rats weaned onto OFS had significantly lower body weight than control diet at weeks 4-18 (P<0.007). Rats weaned onto OFS diet maintained a lower body weight at 19 wk compared to rats weaned onto HE diet but this was no longer significant (P =0.09; Table 4.1). Both body fat (%) and fat mass were significantly lower for rats on the OFS diet compared to HE diet (P <0.004, P<0.007, respectively). There was no significant difference in percent body fat, lean mass, bone mineral density or fat mass between SL and NL rats.

Figure 4.2 Body weight over time

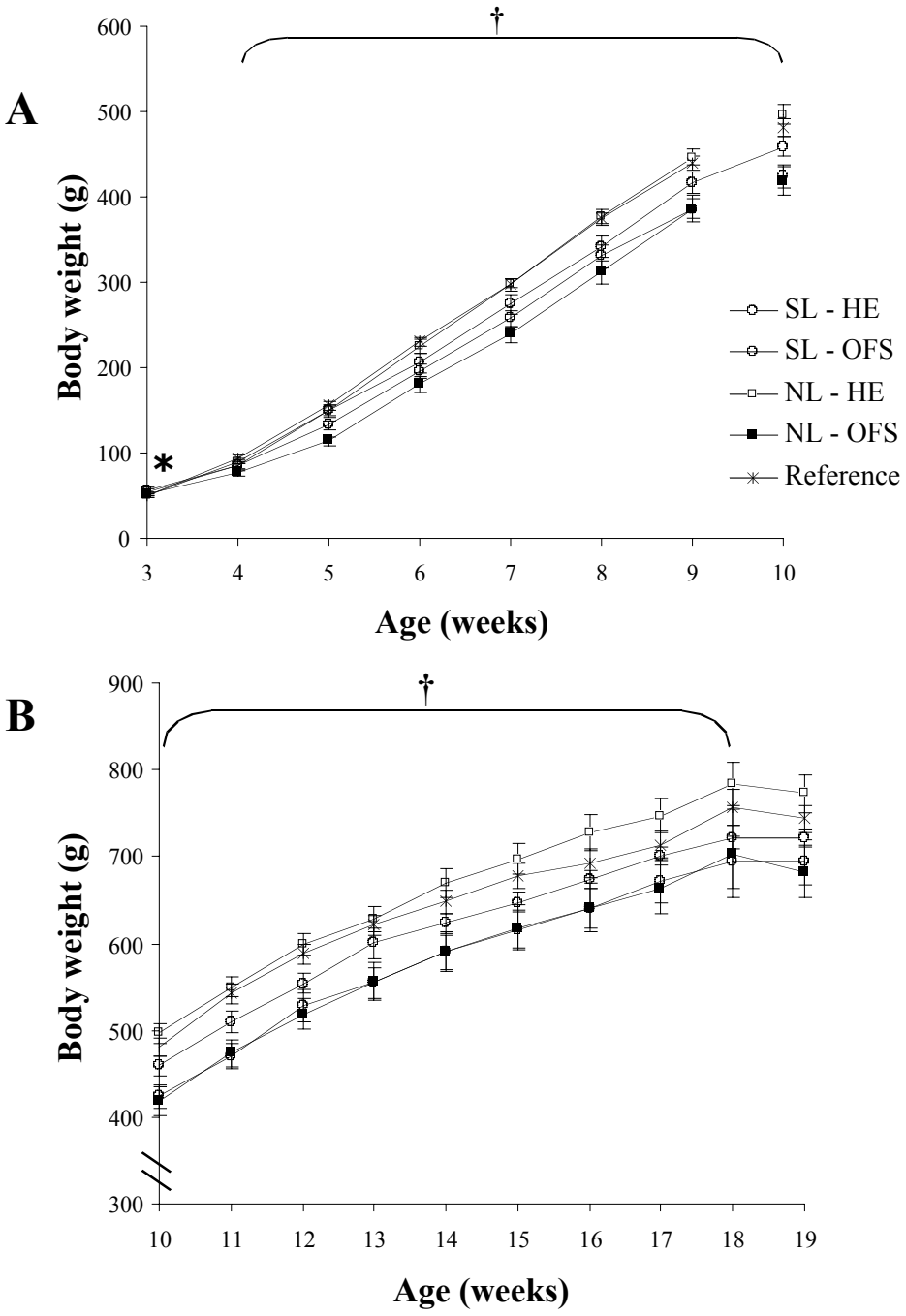


Figure 4.2 Weekly body weight in SL and NL rats fed HE or OFS diet. Panel A represents body weight from weaning (3 wk) to 10 wk of age. Panel B represents body weight from 10 wk to study termination at 19 wk of age wherein the y-axis has been

collapsed to better illustrate group differences. Values represent mean ± SEM (9-10 rats/group). The * represents a significant litter size effect for body weight at weaning between SL and NL rats (P <0.05). The † represents a significant diet effect in body weight between HE and OFS diets (P<0.007). Reference group consists of male rats raised in litters of 12 pups weaned onto AIN-93G (3.8 kcal/g) and at 10 weeks of age were switched onto AIN-93M (3.6 kcal/g). Reference group not included in statistical analysis.

Table 4.1 Body Anthropometric Data

Table 4.1 Body anthropometric data as measured by the DEXA in SL and NL rats weaned onto HE or OFS diets.

REFERENCE [1,2]	**SL – HE**	**SL – OFS**	**NL – HE**	**NL – OFS**	**Litter Size**	**Diet**	**Inter-action**
					2-way ANOVA *P* values		
Weaning body weight (g)	50.4±1.3	54.8±5.0†	56.6±2.3†	49.7±1.4	51.1±2.3	0.032	0.931	0.669
Final body weight (g)	743.4±13.5	720.2±13.7	696.7±24.1	758.0±18.5	702.4±31.8	0.348	0.093	0.488
Body fat (%)	34.9±1.5	35.1±1.8	30.5±1.5*	38.0±2.3	29.6±2.4*	0.631	0.004	0.381
Fat mass (g)	266.8±17.1	253.6±15.3	214.4±17.1*	298.5±24.9	213.0±25.5*	0.327	0.007	0.296

Lean mass (g)	494.1±10.7	466.6±12.0	482.3±11.1	477.4±11.6	489.5±10.5	0.752	0.466	0.540
BMD	0.18±0.001	0.18±0.003	0.19±0.002	0.18±0.001	0.18±0.002	0.215	0.328	0.897

Values are mean ± SEM (n=9-10/group). The † represents a significant litter size effect between SL and NL rats at weaning (P<0.03). The * represents a significant diet effect between HE and OFS diets (P < 0.05). [1]Reference group consists of male rats raised in litters of 12 pups weaned onto AIN-93G (3.8 kcal/g) and at 10 weeks of age were switched onto AIN-93M (3.6 kcal/g). [2]Reference group not included in statistical analysis.

4.3.3 Gastrointestinal anthropometrics

Liver weight was significantly greater in rats from NL compared to SL (P =0.01; Table 4.2). OFS was associated with significantly lower liver weight (P <0.0001) compared to HE. There was no effect of litter size or diet on stomach weight or small intestine weight. Both small intestine length (P <0.001) and colon length (P =0.03) were greater in SL rats and greater with OFS feeding compared to HE (SI, P=0.03, colon, P <0.0001). Colon weight and cecum weight were both significantly greater in the rats fed OFS versus HE (P <0.0001).

Table 4.2 Gastrointestinal Anthropometrics

Table 4.2 Gastrointestinal anthropometrics measured at sacrifice for SL and NL rats on HE and OFS diets.

REFERENCE[1,2]	SL – HE	SL – OFS	NL – HE	NL – OFS	Litter Size	Diet	Interaction
					2-way ANOVA *P* values		
Liver weight (g)	25.4±0.9	21.2±0.5	19.1±0.9*	23.7±0.8†	20.2±0.4†*	0.019	0.001	0.332
Stomach weight (g)	2.9±0.2	2.8±0.1	2.6±0.05	2.9±0.1	2.8±0.2	0.270	0.207	0.363
Small intestine weight (g)	9.5±0.1	9.9±0.4	9.7±0.4	9.8±0.3	9.0±0.3	0.246	0.130	0.454
Small intestine length (cm)	147.6±1.6	145.3±3.7	154.8±1.8*	138.8±1.9†	141.3±3.0†*	0.001	0.032	0.197
Cecum weight (g)	1.1±0.05	1.1±0.08	2.7±0.11*	1.0±0.06	2.7±0.24*	0.798	0.001	0.693
Colon weight (g)	1.60±0.05	1.66±0.08	2.02±0.13*	1.61±0.04	2.13±0.11*	0.732	0.001	0.406
Colon length (cm)	21.8±1.3	23.0±0.6	26.0±1.0*	19.8±1.0†	25.2±0.8†*	0.032	0.001	0.183

Values are mean ± SEM (9-10 rats/group). The † represents a significant litter size effect between SL and NL rats ($P<0.05$). The * represents a significant diet effect between HE and OFS diets ($P<0.05$). [1]Reference group consists of male rats raised in litters of 12

pups weaned onto AIN-93G (3.8 kcal/g) and at 10 weeks of age were switched onto AIN-93M (3.6 kcal/g). [2]Reference group not included in statistical analysis.

4.3.4 Plasma glucose, insulin, amylin and leptin responses

Serial values during the OGTT and total AUC for plasma glucose, insulin, amylin and leptin are presented in Figure 4.3. Rats fed OFS had significantly lower blood glucose levels at time 0, 15, 30 and 120 minutes following the glucose gavage than those fed the HE diet (P =0.002; Figure 4.3A). OFS consumption significantly lowered the total AUC for glucose (P =0.02; Figure 4.3B). There was a significant correlation between body fat (%) and total AUC for glucose (r^2=0.53, P<0.001, Table 4.3, Figure B.3). There was no difference in total AUC for insulin between litter sizes or diets (Figure 4.3D). Rats weaned onto OFS had significantly higher amylin at time 30, 60 and 90 minutes (Figure 4.3E) but did not differ for total AUC (Figure 4.3F). Plasma levels of leptin did not differ between litters or diets. Fasting and total AUC leptin was positively correlated with body fat (%) (r^2=0.34, 0.38 respectively, P=0.04, Table 4.3, Figure B.3).

Figure 4.3 Plasma glucose, insulin, amylin and leptin responses

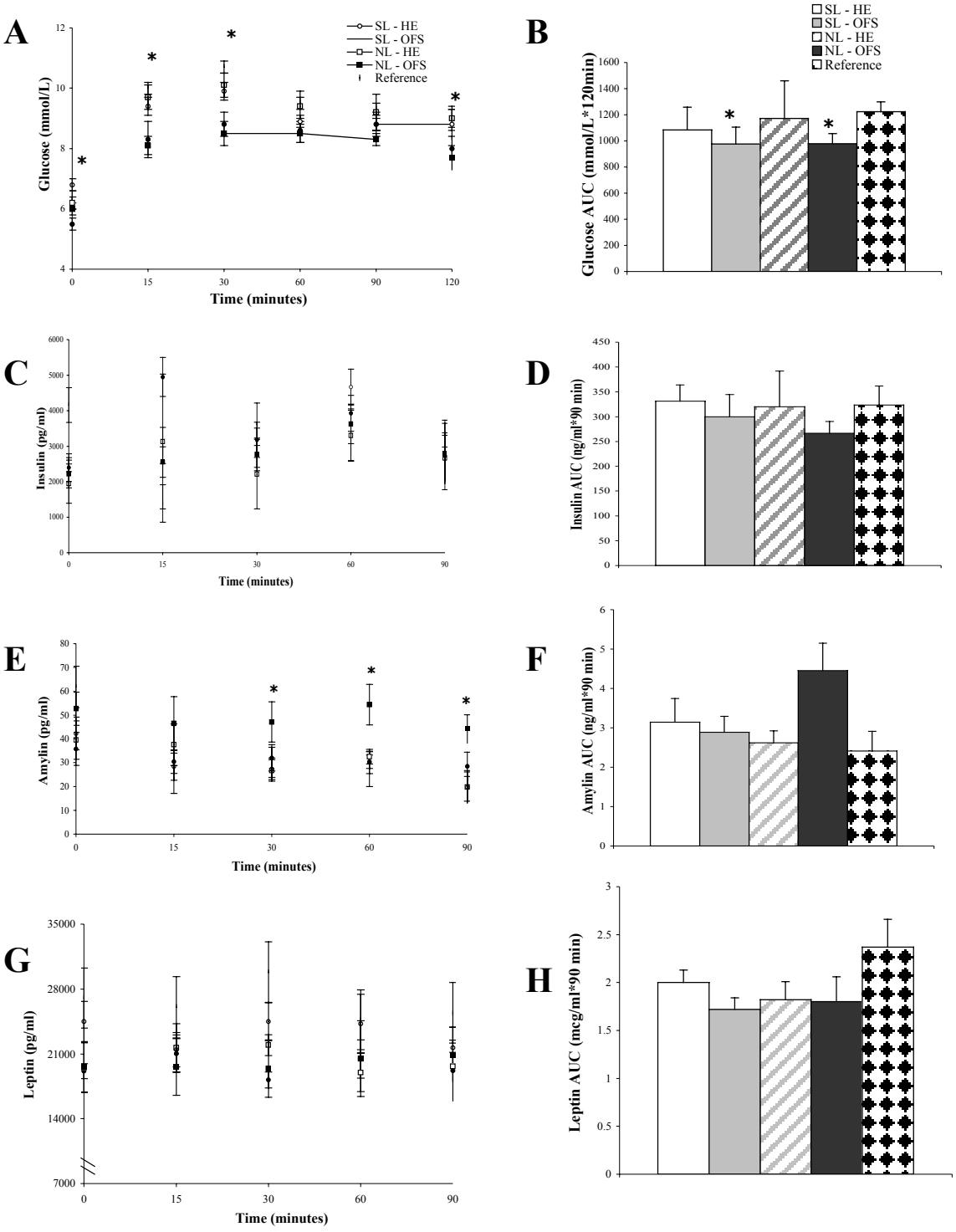


Figure 4.3 Blood glucose, plasma insulin, amylin and leptin responses during a 90-minute OGTT. The left column represents serial values at individual time points and the right column represents total AUC (units adjusted for graphical presentation). Values represent mean ± SEM (9-10 rats/group). The * represents a significant diet effect between HE and OFS diets ($P<0.05$). Reference group consists of male rats raised in litters of 12 pups weaned onto AIN-93G (3.8 kcal/g) and at 10 weeks of age were switched onto AIN-93M (3.6 kcal/g). Reference group not included in statistical analysis.

4.3.5 Plasma GLP-1, PYY, GIP and ghrelin hormone responses

Serial values during the OGTT and total AUC for plasma GLP-1, PYY, GIP and ghrelin are presented in Figure 4.4. The total AUC for GLP-1 was significantly lower in NL rats fed OFS ($P=0.05$; Figure 4.4B). Plasma levels of PYY were significantly higher at time 0, 15, 30 and 60 minutes after the glucose gavage (Figure 4.4C) but total AUC did not reach significance in rats weaned onto OFS ($P=0.069$, Figure 4.4D). Both SL and NL rats weaned onto OFS had significantly lower GIP at time 0, 15 and 30 minutes ($P<0.008$, Figure 4.4E) and lower total AUC GIP in rats weaned onto OFS compared to HE ($P=0.055$, Figure 4.4F). There was a significant correlation between body fat (%) and total AUC GIP ($r^2 = 0.41$, $P=0.02$, Table 4.3, Figure B.3). Plasma levels of ghrelin did not differ between litters or diets (Figure 4.4G & H).

Figure 4.4 Plasma GLP-1, PYY, GIP and ghrelin responses

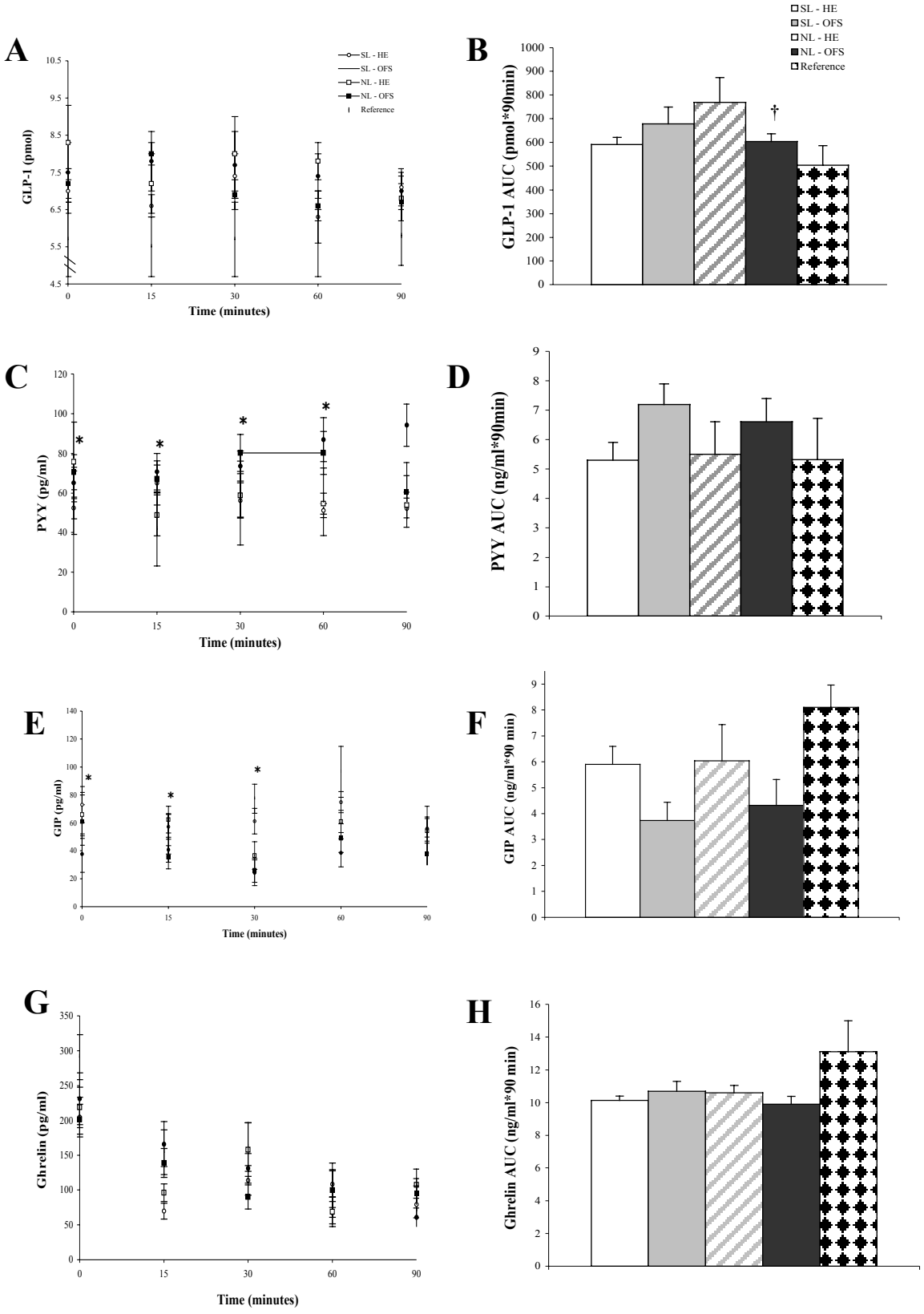


Figure 4.4 Plasma GLP-1, PYY, GIP and ghrelin during a 90-minute OGTT. The left column represents serial values at individual time points and the right column represents total AUC (units adjusted for graphical presentation). Values represent mean ± SEM (9-10 rats/group). The * represents a significant diet effect between HE and OFS diets ($P<0.05$). The † represents a significant interaction between HE and OFS diets for NL rats ($P<0.05$). Reference group consists of male rats raised in litters of 12 pups weaned onto AIN-93G (3.8 kcal/g) and at 10 weeks of age were switched onto AIN-93M (3.6 kcal/g). Reference group not included in statistical analysis.

Table 4.3 Body fat (%) correlations with plasma glucose, insulin and satiety hormones

Table 4.3 Correlation of body fat (%) as measured by DEXA with plasma metabolites at both fasting and total AUC for SL and NL rats weaned onto HE or OFS diets.

	Fasting		**Total AUC**	
	Correlation	***P*-value**	**Correlation**	***P*-value**
Glucose	0.12	0.48	0.53	0.001**
Insulin	0.12	0.52	0.24	0.17
Amylin	0.04	0.84	0.08	0.67
Leptin	0.34	0.04*	0.38	0.04**
GLP-1	0.16	0.40	0.17	0.37
PYY	0.17	0.37	0.05	0.78
GIP	-0.02	0.91	0.41	0.02**
Ghrelin	-0.15	0.40	0.25	0.17

* indicates a significant correlation between body fat (%) and fasting plasma metabolite.

** indicates a significant correlation between body fat (%) and total AUC plasma metabolite.

4.3.6 Hepatic triglyceride and NEFA levels

Triglyceride content of the liver was lower in rats fed OFS compared to HE (P =0.05, Figure 4.5). There were no significant differences in plasma NEFA concentrations between the groups.

Figure 4.5 Liver triglyceride content and circulating NEFA levels

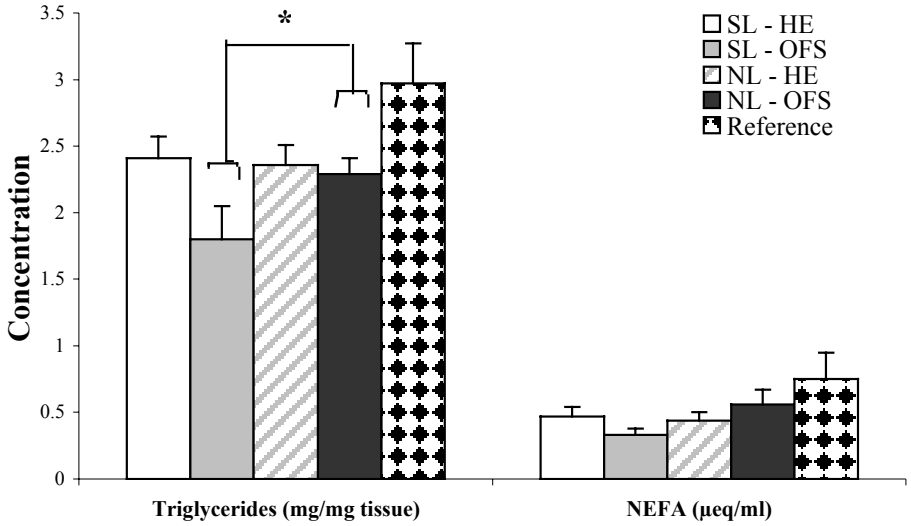


Figure 4.5 Hepatic triglyceride content (mg/mg tissue) and plasma non-esterified fatty acids (µeq/ml) in SL and NL rats fed HE or OFS diet. Values represent mean ± SEM (9-10 rats/group). The * indicates a significant diet effect between HE and OFS diets (P<0.05). Reference group consists of male rats raised in litters of 12 pups weaned onto AIN-93G (3.8 kcal/g) and at 10 weeks of age were switched onto AIN-93M (3.6 kcal/g). Reference group not included in statistical analysis.

4.3.7 Gene expression

In the ileum there was a significant decrease in expression of both proglucagon (P=0.03) and PYY (P=0.03) in rats fed OFS compared to HE diets (Figure 4.6). In contrast, proglucagon mRNA levels in the colon were significantly higher in SL rats versus NL rats (P=0.03) and in rats fed OFS compared to HE diets (P=0.01). SL rats on OFS had a significant increase in PYY mRNA levels in the colon (P=0.01)

In the liver, ACC mRNA levels were significantly higher in rats raised in NL versus SL (P=0.04; Figure 4.7). Although there was a 58% increase in SREBP-1c expression in NL rats over SL rats this was not significant (P=0.09).

Figure 4.6 Small intestine and colon gene expression

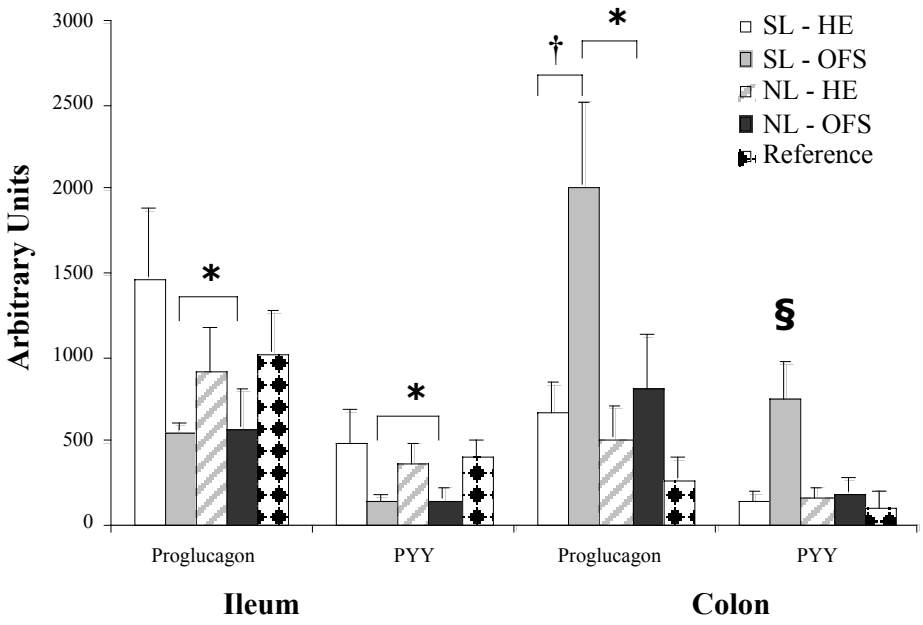


Figure 4.6 Expression of proglucagon and PYY in the intestine of SL and NL rats fed HE or OFS diet. Values represent mean ± SEM (9-10 rats/group). The * represents a significant diet effect between HE and OFS diets (P<0.05). The † represents a significant litter size effect between SL and NL rats (P<0.05). The § indicates a significant interaction for PYY expression in the colon of SL rats fed OFS (P<0.02). Reference group consists of male rats raised in litters of 12 pups weaned onto AIN-93G (3.8 kcal/g) and at 10 weeks of age were switched onto AIN-93M (3.6 kcal/g). Reference group not included in statistical analysis.

Figure 4.7 Hepatic gene expression

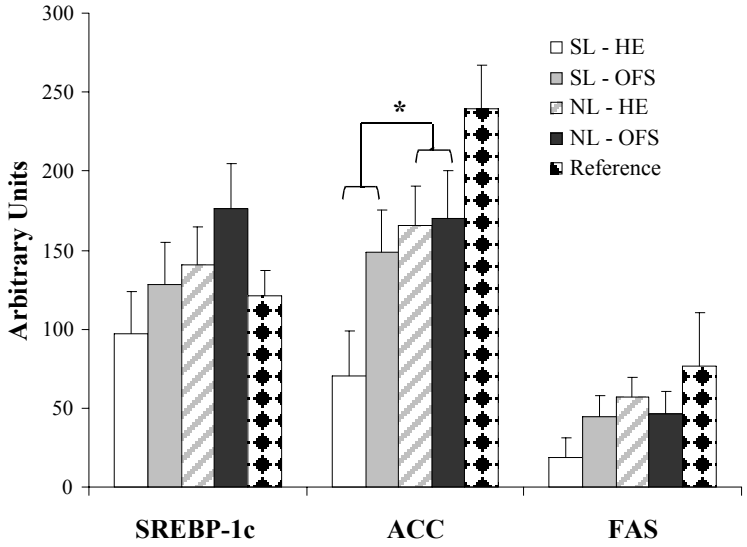


Figure 4.7 Expression of FAS, ACC, and SREBP-1c in the liver of SL and NL rats fed HE or OFS diet. Values represent mean ± SEM (9-10 rats/group). The * represents a significant litter size effect between SL and NL rats (P<0.05). Reference group consists of male rats raised in litters of 12 pups weaned onto AIN-93G (3.8 kcal/g) and at 10 weeks of age were switched onto AIN-93M (3.6 kcal/g). Reference group not included in statistical analysis.

4.4 Discussion

With childhood obesity rates on the rise there is a growing interest in the contributions of early diet and epigenetic factors to the development of excessive adiposity (26). Critical periods in metabolic development have been targeted for investigation to help understand how nutritional status affects body fat accumulation, glucose tolerance and satiety (77). This study examined changes in the metabolism of rats

oversuckled from birth to weaning when randomized to a control (high fat/high sucrose, HE) or OFS-enriched high fat/high sucrose background diet. Although body composition did not vary substantially between SL and NL rats, the SL rats fed OFS displayed mildly elevated GLP-1 and PYY secretion and greater gene expression for these hormones in the colon than the NL rats fed OFS; both changes typically associated with improved body weight control. Overall, OFS feeding appears to moderate the deleterious effects of postnatal high fat/high sucrose feeding and the theory of mismatched dietary influences, discussed in greater detail below, appears to apply to the response of these rats to the diet (88).

4.4.1 Body composition and energy consumption

In accordance with previous literature, the oversuckled pups were heavier at weaning than those raised in normal litters (73). Over the course of the study, however, the predicted changes in body composition imparted by oversuckling seemed to lessen due to the exposure to high fat/high sucrose feeding. Velkoska et al. observed a similar result when oversuckled rat pups were continued on a calorically dense diet into adulthood (24). They showed that after 11 weeks of high fat feeding that there was no longer a litter size effect but a diet effect persisted into 16 weeks. As early as four weeks following birth we detected a significant effect of OFS emerge but no detectible change in body weight linked to initial litter size by the end of the study. It is possible that the high energy diet could have masked or overrode any detectible changes in body composition that might have occurred naturally between the oversuckled rats and those fed a normal caloric density diet. Over the course of our study there was a consistent pattern of lower body weight in rats fed OFS versus control. Given that energy intake was

similar between SL and NL rats, future examination of the metabolic rate of both SL and NL rats may help better inform whether basal energy expenditure varied between the litters.

It is surprising that regardless of litter size rats consumed the high fat/high sucrose diet enriched with OFS at a higher rate than the HE diet alone. Furthermore, SL rats weaned onto OFS seemed to consume slightly greater calories than the NL rats on OFS even when normalized to body weight. In the absence of more detailed experimentation to support this, it appears that the metabolism of the SL rats shifted to better manage the high fat/high sucrose diet when enriched with OFS. Maternal diet studies involving rat pups that were fed the same lard-rich diet in adulthood as was fed to their mothers during pregnancy and lactation had lower plasma glucose and triglyceride levels than pups from the same mothers but fed a control diet in adulthood (10). Although the lard-fed rats also developed hypertension, obesity and hyperinsulinaemia this study presents an interesting case for the theory of adaptive predictive responses (88). From our study it could be suggested that perinatal overnutrition along with high fat/high sucrose feeding in adulthood provides an advantage to these pups in responding to some of the beneficial effects of oligofructose feeding compared to the NL rats. While this seems counterintuitive, it could be attributed to the different amounts of milk received during the suckling period. It has been previously found that oversuckled rats consume 20-30% more milk compared to pups raised in normal sized litter (24). The oversuckled pups may have had the opportunity to consume more beneficial compounds contained in the mother's milk such as hormones, oligosaccharides and immune complexes during the period of weaning which may have lead to a different rate of gastrointestinal maturity and

microbial communities compared to normal-size litters (26, 30, 89). As oligofructose feeding has been linked to changes in strains and numbers of health-promoting bacteria in the large intestine there is a possibility that oversuckled rats were given an advantage during the suckling period that carried into adulthood (41, 79). Assessment of the morphological characteristics of the GI tract and microbial communities present in both the NL and SL rats on HE and OFS diets is warranted to further understand the impact of oversuckling.

Reports from the literature return mixed results for the consequences of matching or mismatching prenatal and postnatal diets. Offspring from mothers fed a high fat diet during pregnancy continued on the high fat diet post weaning had significantly higher fat mass/body mass ratio and plasma leptin levels compared to pups fed a normal energy control diet after weaning (75). In two forms of maternal overnutrition where mothers had diet-induced obesity and type 2 diabetes and were fed a high fat diet during pregnancy and lactation or healthy dams oversuckling pups from birth to weaning, both lead to hyperphagia and glucose intolerance (90). In another case, there were no postnatal diet effects in offspring from pregnant mice fed either normal or restricted protein diets weaned onto a high fat diet although all mice on the high fat diet became heavier than control mice (91). Indeed prenatal or postnatal or combined high fat feeding regimes will lead to deleterious metabolic consequences, however, from our study it appears that continuing oversuckled pups onto a higher calorie diet into adulthood allowed them to respond better in certain metabolic parameters elicited by high fiber feeding compared to the NL rats. A future consideration in this area might involve teasing out whether or not

matching the dietary environment from birth to weaning compared to weaning onwards plays a role in influencing longer-term metabolism.

4.4.2 Satiety hormone response

Since OFS feeding is known to increase GLP-1 response, the increase in total AUC for GLP-1 was anticipated in SL rats between HE and OFS diets (41). Even though the NL-HE rats had almost a two-fold greater secretion of GLP-1 than SL-HE rats, NL-OFS rats demonstrated a decrease in GLP-1 secretion below that of SL-OFS rats. Although unexpected, this result may be explained in part by differences in milk exposure during the suckling phase limiting the NL rats' reaction to the stimulus of OFS (24). Changes in nutritional provision during the early postnatal period have been linked to alterations in tissue development and molecular signalling in rats (77). For example, exchanging the normally high fat content of rat milk for carbohydrates has been shown to precipitate hyperinsulinaemia and structural changes in pancreatic islet cells (72, 92). Additionally, rat pups suckled by dams with gestational diabetes had altered appetite signalling in the hypothalamus which resulted in adult animals that were obese (77). Increased access to nutrition during suckling may have predisposed the SL rats to the positive satiating effects of OFS, however, this scenario does not adequately explain the NL rats' lower secretion of GLP-1 while consuming the high fat/high sucrose diet so further study is clearly warranted.

Although the secretion of PYY in NL rats did increase with OFS, the SL rats had greater total AUC on OFS than the NL rats. One explanation for the discordance in GLP-1 and PYY secretion between NL rats and SL rats could be epigenetic changes imparted in the SL rats during the period of overnutrition between birth and weaning (68). SL rats

had a significant increase in the expression of proglucagon (coding for GLP-1) in the colon as well as SL-OFS rats had significant upregulation of PYY in the colon. A change in gene expression for certain gastrointestinal enzymes has been shown to result from an increase in sustained transcriptional activity (93). Epigenetic influences in the maturation of the gastrointestinal system have also been demonstrated in the increased incidence of colorectal and duodenal cancers in family members who have overexpression of certain genes (30). It is possible that the extra nutrients absorbed from the mother's milk may have promoted the expression of certain gastrointestinal genes leading to a greater sensitivity to the satiating effects of OFS.

4.4.3 Glucose response

The positive correlation between body fat (%) and glucose total AUC further confirms the glycemic regulatory benefits of OFS consumption (94). Even on a high fat/high sucrose diet, OFS feeding led to a decrease in both body fat and glucose total AUC and interestingly both the SL and NL rats responded in a similar manner to the fiber feeding. We previously found a positive correlation between body fat and glucose total AUC in rats fed a normal calorie diet enriched with OFS, however, the SL rats had mildly increased body fat and glucose total AUC with OFS feeding whereas the NL rats had both a decrease in body fat and glucose total AUC with the fiber. This continues to support the theory that oversuckled rats fed a high fat/ high sucrose diet retain the capacity to respond positively to OFS feeding compared to oversuckled rats who when weaned onto a lower calorie diet do not display the positive metabolic effects elicited by OFS feeding.

4.4.4 Lipid metabolism

OFS supplementation consistently led to lower hepatic triglyceride levels in both SL and NL rats. Previously we found that SL rats raised on a normal calorie diet with OFS responded with higher triglyceride content in the liver whereas NL rats had lower liver triglyceride content. The exact mechanisms of lipogenesis are not fully understood and there are reported differences in the levels and activities of enzymes responsible for lipogenesis between rodent models and humans. Delzenne et al. found decreased liver triglyceride content in adult rats fed a diet supplemented with 10% oligofructose (by weight) (78). They linked lower hepatic triglyceride and circulating very-low-density lipoprotein (VLDL) levels to reduced hepatic triglyceride synthesis due to decreased lipogenic enzyme activity including ACC and FAS. Decreased FAS expression in visceral adipose has also been observed in obese subjects fed a low fat-high sugar diet (95). Without further study is it difficult to determine if the decrease in hepatic ACC expression without a proportional decrease in FAS in our SL rats is responsible for the decreased triglyceride synthesis and was a potential response of the dietary programming. Currently there is considerable focus on increased FAS expression contributing to insulin resistance however there is very limited research linking FAS and ACC expression to metabolic dysregulation (96). It is interesting to note that differences in FAS enzyme activity have been detected in high fat fed mice between fasting and fed conditions indicating that it may be necessary to assess the lipogenic enzyme activity in both states (93). Continued study of the mechanisms related to lipid metabolism in these animals is required.

4.5 Conclusion

This work provides evidence that oversuckled rat pups fed high fat/high sucrose diet from weaning have the ability to respond to some of the metabolic effects of OFS supplementation. Further work is required to understand what mechanisms may have increased the capacity of oversuckled rat pups to respond to prebiotic fiber feeding. Examination of the microbiota profile of both SL and NL rats along with SCFA production may help elucidate these mechanisms.

One theory that emerged from studying the response of oversuckled rats on OFS was that the potential consequence of caloric imbalance between the energy density of diets from the period of birth to weaning compared to weaning onward and the 'predictive adaptive response' that may have been elicited in these animals (88, 97). Previous work by Khan et al. demonstrated that fat-fed male offspring from dams fed a high fat diet during pregnancy and lactation had lower circulating glucose and less endothelial dysfunction compared to rats weaned onto standard chow (87). The authors suggested that the animals had the capacity to adapt their metabolism *in utero* to deal with the dietary insult received after birth and prevent certain debilitating effects of high-fat feeding.

Further work considering the metabolic consequences of a nutritional imbalance between the suckling period and adulthood is required. Studying the interaction between epigenetic programming and dietary influences may provide a greater understanding of how these factors contribute to long-term metabolism. As well, with this understanding we may be able to provide further evidence for dietary guidelines in order to prevent excessive childhood weight gain and adulthood obesity.

Chapter Five: Discussion

The main focus of these studies was to quantify the metabolic response elicited in oversuckled rats fed oligofructose compared to control diets. To assess the primary outcome measure, body fat accumulation, a DEXA scan was performed. In addition, satiety hormones in the plasma were analyzed along with mRNA expression in segments of gastrointestinal tissue responsible for the transcription of these hormones. A study of triglyceride metabolism concluded this initial assessment of the influence of prebiotic fiber feeding in oversuckled rats.

Overall it was found that oversuckled rats respond to oligofructose feeding differently than rats that have been raised in normal sized litters. Also it appears that the diet that the rats continue to consume following weaning plays a role in the capacity for the rats to respond to the positive effects of oligofructose. The following chapter highlights the major findings from these studies as well as suggests future research directions. Strengths and limitations of the studies are also discussed.

5.1 Strengths and Limitations

5.1.1 Oversuckling model

The oversuckling model was chosen for this project as it had been previously shown to result in overweight pups that exhibit metabolic disturbances including excessive adiposity, hyperglycaemia and hypertension once these rats reached adulthood (17, 21, 74). This is of current importance since both adult and childhood obesity rates are on the rise (3). Although it must be recognized that infants are born at a different stage of development than rodents, infants still retain a certain amount of plasticity after

birth (7). With this in mind we looked to further understand how postnatal nutrition influenced these developing metabolic structures.

A detailed literature search returned many studies that had used the oversuckling model (11, 17, 20, 21, 24, 26, 74, 89, 98, 99). However, when comparing the litter sizes that had been employed it was found that they varied anywhere between 2-8 pups/litter. As well, normal litter sizes ranged from 8-24 pups/litter. Based on the previously published work we decided on 3 pups/litter for our oversuckling model since studies of this size reported metabolic abnormalities consistent with those observed in overweight and obese children (24). For age-matched controls a normal litter size of 12 rats was chosen, as this is close to the average size of rat litters and would still be achievable given any pup loss in the first few days following birth. Although the variation in litter sizes somewhat limits our ability to generalize the findings to similar studies in the literature, having the normal litter counterparts allowed us to compare our findings against rats raised in a normal suckling environment.

Our litter manipulation protocol followed the method outlined by Velkoska et al. in which litters of pups were randomly adjusted to SL or NL sizes approximately 24 hours after birth (24). In contrast, studies using the oversuckling model from the Plagemann group have followed a method of randomly distributing all pups to different mothers for the first three days after birth before manipulating the litter sizes to 3 or 12 pups at day three post-partum (76, 99). It is possible that the differences in the initial rearing practices of the pups could have induced the long-term metabolic disturbances observed in the results of the Plagemann group compared to the body composition results found in both our study and the work published by Velkoska et al. (24). In addition to these potential

rearing differences, it would also be valuable in future studies to assess the composition of the mothers' milk throughout the suckling period to determine macronutrient content and resulting nutrient absorption in the pups that could play a role in long-term metabolic programming.

Another consideration to make regarding the differences between the SL and NL rats is the variation in nest sizes during the suckling period. There is a possibility that the metabolic rate of oversuckled pups was higher because they needed to burn more calories to sustain their body temperature compared to the 12-pup litters that could used their combined body heat to stay warm. This may have changed the rate that the oversuckled pups stored excess energy and as a result may have confounded the obesity programming imparted to the pups during suckling.

5.1.2 Double-Hit Hypothesis

Our study varied from previously published work since we received pregnant rats into our facility compared to breeding the dams in our facillity. Examples from the literature have demonstrated deleterious psychological and endocrine effects in rats that are exposed to stress *in utero* followed by a second application of stress during critical periods following birth (100). Known as the 'double-hit hypothesis' it has been suggested that prolonged exposure to environmental stressors at a young age and again in childhood or adolescents can lead to adverse changes in mental function ad hormonal responses (101). Since the dams used in this study were exposed to some stress during shipping it is possible that the oversuckling provided a second hit to the pups and may have been responsible for some of the metabolic changes observed. In the future it would be

interesting to breed the stock in-house to see if there are any differences detected in the metabolic variables measured in the current study.

5.1.3 Physical Activity

Although physical activity between the different rat groups was not assessed in this current study in the future it may be valuable to quantify the pattern and duration of the rats' daily activity. Changes in physical activity due to the rearing practices employed in this study may have influenced the metabolic rate between the groups making it difficult to determine the sources of any changes in body composition.

5.1.4 Diets

There are a number of factors that may have influenced the growth of the rats based on their diets. The powdered AIN-93 diets fed to the rats is a highly refined mixture that varies substantially from the diet a rat would consume in nature. Dietary studies using rats often feed a pellet-type chow that is only semi-purified compared to our purified rat diet used in this study. As well, we have found in our laboratory that control groups of Sprague-Dawley rats raised on AIN-93 powdered diet became the heaviest over time. This coupled with the different diet may have lead to the unexpected body weight changes observed in this study.

5.1.5 Sample size considerations

Based on ascertaining a 20% change in GLP-1, the sample sized required for this study was 6 rats/diet group. Accounting for potential loss throughout the study, a sample size of 9 rats/diet was entered into the study. We have previously had rat loss in our studies due to unexplained infections, fighting resulting in injuries and/or death, and a twisted cecum, likely the result of an adverse reaction to dietary fiber (albeit at double the

dose utilized in this study). Rats lost in this study were all from the normal sized litters and of those lost two were due to events unrelated to the dietary intervention, two were lost due to extremely high blood glucose levels during the OGTT and three were lost due to unidentifiable reasons (veterinary pathology report unable to identify cause).

5.1.6 Oral glucose tolerance test

An OGTT was selected over a meal tolerance test (MTT) to examine the plasma glucose, insulin and satiety hormone response. Previous work in our lab has found that using voluntary meal consumption in the rats is very challenging and leads to inconsistent amounts of test meal consumption by the rats in a defined time period, thereby completely confounding the results. In order to limit this confounding effect, an OGTT using a standard dose of glucose per kilogram of body weight was used to assess glucose tolerance and satiety hormone levels. The OGTT worked well to assess the glycemic response in our rats, however the satiety hormone response was less robust and did not display a strong rise and fall following the glucose load. Similar plasma glucose results were found in Zucker rats during an OGTT but a MTT proved to elicit a greater insulin response than the OGTT (102). In future work, a standard liquid diet such as Ensure may provide a consistent and easily administered meal comprised of carbohydrates, protein and fat to all rats in order to elicit a more physiologically relevant satiety hormone response over the OGTT alone.

5.1.7 Body composition

A DEXA machine was used to assess the whole-body composition of the animals. Although this scan does not indicate the distribution of the body fat of each animal it provided us with a more accurate estimate of total fat mass and lean mass compared to

site-specific fat pad dissection (103). Although an in-direct measure of body composition, DEXA is the gold-standard for this assessment (104). With the DEXA scan we are also able to ascertain the bone mineral density of each animal and relate it to the dietary intervention which is of interest in our studies given the role of prebiotics in increasing intestinal calcium absorption and improved bone health (105).

5.1.8 Plasma hormone testing

The plasma satiety hormones were determined using a multi-analyte sandwich assay with fluorescent-labelled microspheres from Millipore. Since the volume of plasma that can be collected from each animal was limited, assessing multiple hormones at once in a small volume of blood allowed us to study a broad spectrum of hormones that would not normally be available.

Due to limited sensitivity at the low end of the standard curve we were not able to detect changes in GLP-1 using the Milliplex kit. Instead a commercially-available ELISA kit was used to assess this hormone. In the future, direct assessment of the GLP-1 present in ileum and colon tissue could be assayed using the ELISA kits to determine the amount of GLP-1 present in the tissue compared to proglucagon expression coding for GLP-1 in addition to other peptides. Plasma samples were aliquoted in triplicate to allow for assessment with both the Milliplex assay and the ELISA without putting the plasma through additional freeze/thaw cycles. Since we looked at plasma values across multiple time points in each rat that did not fit in one single plate we created Bland-Altman plots in order to ensure reliability between each plate (106). We found that ghrelin, PYY and insulin had acceptable reliability but in the future we may want to consider ELISA kits

for GIP, amylin and leptin in order to confirm strong reliability between plates (Figures C.1 and C.2).

5.1.9 Quantitative real time-PCR

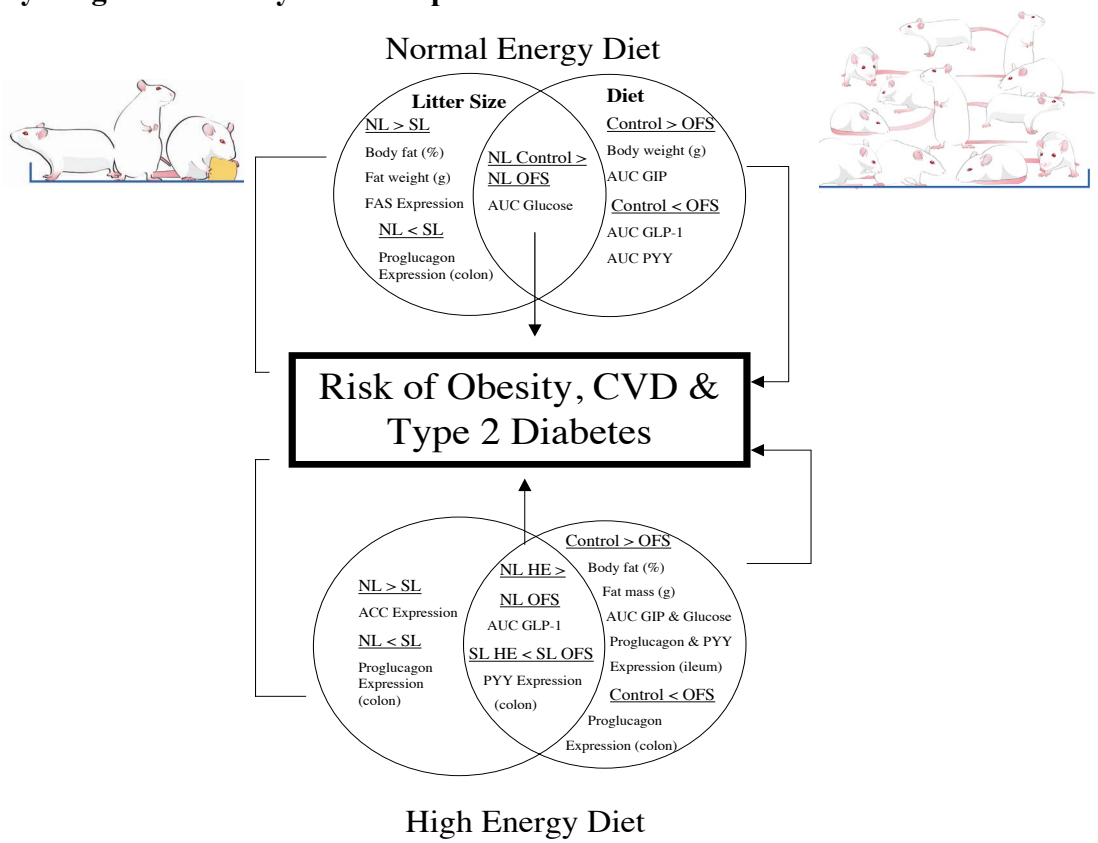
In order to determine the reliability between different qRT-PCR plates Bland-Altman plots were made based on the method determined by Bland et al. (106). There was satisfactory agreement between plates (Figures C.3 and C.4) which increases our confidence that mRNA levels obtained from one plate were in agreement with those from additional plates when the number of related samples were too high to fit on one plate. It should be noted that there was a trend for greater variability when higher mRNA levels were detected.

5.2 Global Interpretation and Discussion

5.2.1 Summary of major findings

Figure 5.1 below represents a schematic of the independent and collective effects of litter size and oligofructose supplementation on important metabolic outcomes related to obesity. It is apparent that early dietary influences play a role in determining life-long metabolic health. Continued work in the area has the potential to drive changes in both prenatal and postnatal dietary guidelines in order to help slow down the development of obesity. The current findings may help in determining why certain individuals retain the capacity to respond to dietary weight-loss interventions more successfully than others. It is clear that nutritional status during many critical periods has the potential to contribute to preventing obesity and related comorbidities.

Figure 5.1 Schematic of independent and interactive effects of litter size and diet on body weight and satiety hormone production in rats



5.2.1 Body composition

There were significant changes in body mass between rats fed OFS or control dependent upon whether rats were fed a background diet of normal calorie density standard ration or high calorie density high fat/sucrose diet. On the normal energy diet, the SL rats responded to the OFS-feeding with reduced body weight however surprisingly, their percent body fat and fat mass, arguably the more important indicators, did not reflect the positive changes that were observed with the NL rats. Although not reaching significance, the liver triglyceride levels in the SL rats on OFS increased compared to the control-fed rats. Also surprising was the decrease in lean mass in the SL rats on OFS compared to the control diet. These results taken together seem to indicate

that oversuckling from birth and weaning likely influenced lipid metabolism in these animals.

One theory that may help explain these changes considers the marked disparity in increased calorie consumption between oversuckling and then the relatively lower calorie diet at weaning. This is based on the premise that rat milk has a relatively high fat content and when the pups from small litters suckle they overall consume more fat in proportion to protein and carbohydrates (107, 108). When these animals are then switched to a lower calorie diet at weaning, even lower in rats fed the diets enriched with oligofructose, they may respond by conserving calories in adipocyte fat stores.

A similar relationship can be applied to the animals weaned onto the high energy diets. However in this case it appears that both the SL and NL rats responded to the OFS by accumulating less fat mass while maintaining lean body mass. Without the drastic change in caloric content of the diets the SL rats responded to the fat modulating effects of oligofructose similar to those of the NL rats.

As the consumption of mother's milk in humans has been shown to be not inherently harmful but instead provide a boost to the immune system and metabolic protection it is instinctive that the oversuckling per se is not as metabolically damaging as the long-term diet effects (16, 109). In order to test this theory further analysis of the dams' milk while the animals are suckling is imperative. As well providing the dams with a high energy density diet along with oversuckling may mimic more deleterious consequences than just oversuckling alone. It could be theorized that mismatching the dietary environment from birth to weaning compared to weaning onward may be of more consequence than the oversuckling by itself.

5.2.2 Satiety hormone response

The prebiotic fiber feeding elicited an increased secretion of GLP-1 in both SL and NL rats fed a normal calorie diet. Additionally, SL rats on both normal energy and high energy diets had a significant upregulation in proglucagon in the colon on OFS. These findings seem to indicate that there may be a positive relationship between oversuckling and increases in L-cell proliferation and secretory products in the colon. However the overall statistical increase in proglucagon expression did not translate into a significantly greater secretion of GLP-1 in the plasma in SL rats. Whether this represents a defect in L-cell secretory machinery in these animals is not known. Further study of upstream products including other peptides produced by proglucagon and post-transcriptional modifications of the products including GLP-1 may help in clarifying this relationship.

A different relationship in GLP-1 secretion was found between NL and SL rats on the high energy diet. As expected the high fiber feeding led to an increase in GLP-1 secretion in the oversuckled rats however we noticed a decrease in the secretion in NL rats. It is possible that the increase in milk received by the oversuckled rats primed their gastrointestinal system to the benefits of oligofructose. These findings combined with the body weight data seemed to indicate that the post-natal nutritional environment plays a key role in predisposing the gut to be sensitive to the positive effects of oligofructose.

5.2.3 Blood glucose and insulin responses

Both groups of NL rats fed NE and HE diets had positive glucose and insulin responses when fed OFS. With the fiber enrichment the total area under the curve for both glucose and insulin decreased. However a surprising relationship was found for the

SL rats from the NE diet group. Rats weaned onto OFS had greater area under the curve plasma glucose than control fed SL rats. This unexpected result will require further study to determine if it was due to the early dietary programming.

5.3 Major Outcomes in relation to the Literature and Future Directions

5.2.4 Epigenetic alterations

With increased focus on childhood obesity early nutritional changes have been linked to the promotion of gene expression changes within the field of epigenetics. In general, terms such as “nutritional programming”, “imprinting” and “developmental plasticity” have all been used to describe the influence of the nutritional environment on certain heritable mitotic alterations in gene expression that do not result in permanent changes to the DNA (15, 26, 30, 110). Stemming from clinical studies associating abnormal levels of circulating steroid hormone levels with adverse neuroendocrine development, the study of peptide neurohormones and resultant metabolic regulatory system changes are also being considered (110, 111).

5.2.4.1 DNA methylation

One of the potential mechanisms that have been identified to be responsible for the metabolic disturbances observed in oversuckled pups involves structural changes in the central nervous system (99). Of particular interest are the orexigenic, appetite stimulator NPY and the anorexigenic, appetite suppressor POMC both released from the arcuate nucleus of the hypothalamus (24, 99). At this point the literature findings indicate that the expression of these neurohormones seems to be protected to a different extent following adverse nutrition.

In one case, oversuckled rats fed a high fat diet had lower concentrations of NPY in the paraventricular nucleus (PVN) compared to normal chow fed animals (24). Alternatively, NPY promoter regions were found to have equally low levels of methylation in oversuckled and control rats at 21 days of age (99). Although we did not study these neuropeptides directly we did identify in the oversuckled pups a significant increase in plasma levels of PYY, a known competitor to NPY at the Y2 binding site in the hypothalamus without seeing the anticipated significant reduction in food intake. This may indicate that although the oversuckling treatment did not lead to significant changes in NPY expression there may have been a disruption in the interaction between NPY, PYY and the Y2 receptor resulting in sustained levels of food consumption and body fat in the oversuckled animals. Future work is needed to further elucidate if oversuckling induces changes in NPY in relation to the sensitivity to PYY and appetite regulation.

Contributing to adverse appetite control is increased methylation of the POMC promoter regions causing a decreased release of POMC in the arcuate nucleus of the hypothalamus leading to a lack of centrally-controlled appetite regulation (99). In this case these animals may be resistant to the neural satiating influence of insulin and leptin (99, 112). Following up on changes in both central appetite stimulation and control may further our understanding of the long-term consequences of adverse perinatal nutrition.

5.2.4.2 Early origins of disease hypothesis

Deleterious metabolic function has been identified in children who were born small for gestational age but were nutritionally supplemented after birth (110). This phenomenon is known as ‘catch-up growth’ and can be related to epigenetic changes induced during inadequate prenatal nutrition mismatched with a plentiful one after birth

(11, 113). Increasing rates of obesity in developing nations has contributed to the realization that a U-shaped relationship between birth weight, either low or high, is related to increased risk of metabolic dysfunction in adulthood (15, 113). This presents part of the puzzle, where prenatal and early postnatal nutrition contribute to the increased risk of metabolic diseases in later life, however, further environmental strain on these compromised systems must be considered. These observations have been extended to include 'predictive adaptive responses' related to a mismatch between the chosen early-life nutritional track and an obesogenic environment in later life (88). Targeting these mismatched situations provides evidence for reversal of this paradigm. As discussed earlier, postnatal leptin injections following *in utero* growth restriction reversed the deleterious effects of the prenatal programming (66). It is suggested that the increase in leptin signalled a positive energy balance and led to decreased food consumption, essentially matching the energy intake to the prenatal environment. Based on our results we further suggest that these responses could be extended into the postnatal period in rats. Weaning oversuckled rats onto the high energy diet constituted a more similar diet to their pre-weaning diet than the normal calorie diet allowing the high fat fed rats to respond to a greater extent to the glucose lowering effects of oligofructose. This provides evidence that future childhood dietary guidelines may need to take into account both the prenatal and postnatal dietary experiences when advising for optimal health.

5.2.5 Maternal contributions

5.2.5.1 Maternal hyperglycaemia

Pre-gravid women with diabetes and those who have uncontrolled gestational diabetes are at high risk of giving birth to a hyperinsulinaemic and hyperleptinaemic

infant (15). Similarly, oversuckled pups assessed at 21 days of age are also found to be hyperinsulinaemic and hyperleptineamic (110). It has been suggested that these supraphysiological levels of hormones could act as endogenous teratogens and influence both central and system tissue development and regulatory processes (110). In animal studies assessing the generational effect of maternal type 2 diabetes, spontaneous hyperglycaemia has been observed as far as the third generation (112). Remaining to be determined is if the increased insulin and leptin levels observed in rat pups are a direct response of the oversuckling or if there are other mechanisms influencing these elevated plasma levels.

An important distinction to make is the differences in the development time frame between altricial species such as rodents and precocial species including human infants (89). Rodent species are born early in maturation and there is a fair amount of growth and development in both the cardiovascular and endocrine systems that takes place after birth including an observed leptin surge at about day 10 post-partum (66, 114). Infants however are born at a much more mature stage in their development. Taking into account these different stages of development many rodent models have been designed to assess the effect of varying adverse dietary treatments (10, 11, 21, 86). Often children born to mothers with gestational diabetes are large and are hypoglycemic with an increased risk of type 2 diabetes in adulthood and these factors have been linked to placental changes *in utero* (31). An important distinction to make is that although oversuckled rat pups are hyperinsulinaemic and hyperleptinaemic at weaning, the source of those changes may be different than pups born to a mother with diabetes or was fed a high carbohydrate diet during pregnancy as the presentation of these plasma markers may be due to different

aetiologies. Further work is needed using these models to determine more precisely what each model represents in reference to human growth and development. Rodent models of maternal hyperglycemia may prove to be more successful in determining the resultant changes in offspring whereas oversuckling pups from birth to weaning may indicate metabolic changes resulting from poor postnatal nutrition. In order to determine if gestational hyperglycaemia could be prevented in these cases future work could focus on the capacity for oligofructose supplementation to promote glucose tolerance during gestation.

5.2.6 Gastrointestinal integrity and immunology

5.2.6.1 Gastrointestinal changes

Of key interest with the oversuckling model is the relationship between postnatal oversuckling and gastrointestinal maturity (115). A full-term neonate has a structurally developed immune system but severely lacks functional adaptive immune responses (116). Contributing to the maturity of the immune system are bioactive ingredients contained in mother's breast milk (53). In both the human neonate and young rat pup similar changes in the gut development in response to suckling have been observed. Decreased gut permeability, increased height of crypts in the small intestine and increased activity of lymphatic tissue have been associated with maternal milk feeding (53). In a newly born rat pup only gram positive bacteria have been found and in the human neonate breast feeding seems to contribute to a decreased CD4+:CD8+ ratio and increased natural killer (NK) cells (116). With the introduction of solid foods, the gastrointestinal tract is challenged with various new strains of bacteria and toxins indicating the importance of a mature gut to combat or develop tolerance to these

challenges (109). Use of the oversuckling model to further track changes in gut maturity from birth to weaning may assist in understanding how nutritional provision from the mother may aid in decreasing metabolic enterotoxaemia and related autoimmune disorders (83).

5.2.6.2 Gut microbiota

In addition to the effects of satiety hormones, the colonization of microbiota in the gut plays a role in the absorption of energy from food (83). The discovery of gut microbiota involvement in energy harvest from food led to the identification of differential proportions of two main classes of bacteria in lean versus obese animals, the Bacteroidetes and Firmicutes (117). First characterized in mice, Ley et al. found a 50% reduction in Bacteroidetes and proportional increase in Firmicutes in obese mice compared to lean controls (117). A study looking at the microbiota of children showed that children who harboured a higher count of bifidobacteria as young as one year old were leaner at 7 years compared to children who had lower counts of that bacteria (118). Certain non-digestible carbohydrate sources such as inulin and oligofructose have been shown to reverse the effects in an obese gut by the cultivation of beneficial bifidobacteria and lactobacillus strains to match the profile of a lean individual (79). From the current study differences in body weight and satiety hormone gene expression indicate that there may be a relationship between oversuckling and the response to oligofructose supplementation. Future studies will work towards identifying the bacterial groups and profiles of microbiota present in the cecal contents of oversuckled rats versus NL control rats. This information will further our understanding of the influence of short-term early post-natal feeding on longer-term gut microbiota health.

5.2.6.3 Gastrointestinal integrity

Colonization of bifidobacteria is important, as it has been shown to control pathogenesis by lowering the gut pH, secreting antibiotic-like substances and protecting against highly invasive pathogens (68). Strategies to promote optimal/stable microbiota in infants are fostered by promoting breastfeeding for at least four months and more ideally for six months (119). However, given the many challenges faced by mothers in achieving this target there is an emergence of infant and toddler food products that are enriched with prebiotic fibers (67). These non-digestible carbohydrates increase the growth of beneficial bacteria especially when the gut has been inoculated with health-promoting bacterial strains such as bifidobacteria (68). Most infant formulas that are supplemented with prebiotic fiber use a combination of inulin and galactooligosaccharides (10% and 90% by weight, respectively). In both preterm and term infants, randomized controlled trials in which 28 days of prebiotic supplementation (0.8g/day) resulted in similar bacterial profiles to the control breastfed groups (116). Unknown at this point is the ability of prebiotic supplementation to bring about sufficient changes in gut microbiota to be protective against pathogens or of relevance to our work and the obesity epidemic to provide protection against excess adiposity development in the long term. There is potential for the integrity of the gut to be higher with breastfeeding because of the contributions of innate immunity complexes transferred from the mother to the child via breast milk (116). This advantage may lead to a decreased risk of developing autoimmune diseases including type 1 diabetes (109, 116).

5.2.6.4 Biomarkers

Changes in nutritional status during fetal development have lead to the characterization of long-term metabolic consequences (120). Markers of some of these traits include risk factors for heart disease, diabetes and hypertension and can be detected using biomarkers such as blood cholesterol and fibrinogen (31). Based on the evidence of hyperinsulinaemia in infancy contributing increased risk of type 2 diabetes in adulthood work is needed to determine if this would be a potential biomarker that could used to identify children who are at increased risk of developing metabolic diseases in the future (107). Increased glucocorticoids concentrations in adipose tissue of oversuckled rats have been linked to obesity, glucose metabolism and blood pressure (74). Determination of these levels in children may indicate the susceptibility for type 2 diabetes in the future and should be looked at as a potential biomarker (98).

5.3 Conclusion

Overall these studies indicate that oligofructose supplementation is able to reduce body weight gain and glucose levels independent of litter size for rats. Furthermore, we were able to detect differences in response level of OFS in SL rats when weaned onto a normal energy diet versus a high energy diet. Potential areas for further research include assessing differences in cecal microbiota and changes in lipid metabolism between litter sizes and diets. The obesity epidemic is one of the largest looming global health crises (2). By understanding the potential for targeted dietary interventions that are based on widely acceptable food ingredients/dietary patterns to mitigate susceptibility to obesity, this work is likely to spur future investigations of this nature.

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APPENDIX A

Figure A.1 Change from baseline for glucose, insulin and satiety hormones

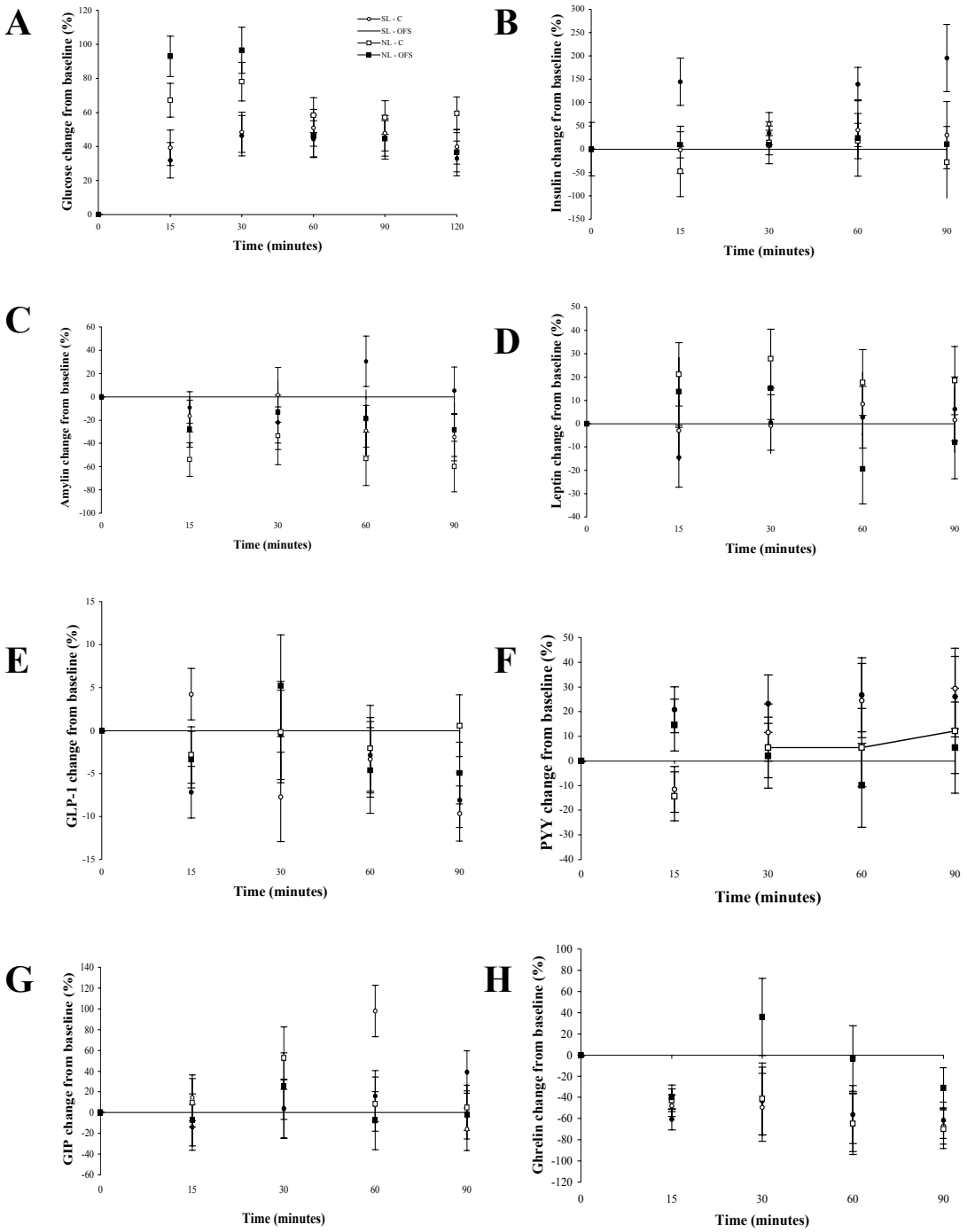

Figure A.1 Change from baseline (%) for glucose, insulin and satiety hormones in SL and NL rats weaned onto C or OFS diet during an OGTT. Values represent mean ± SEM (9-10 rats/group).

APPENDIX B

Figure B.1 Body fat (%) correlations with glucose and leptin total AUC

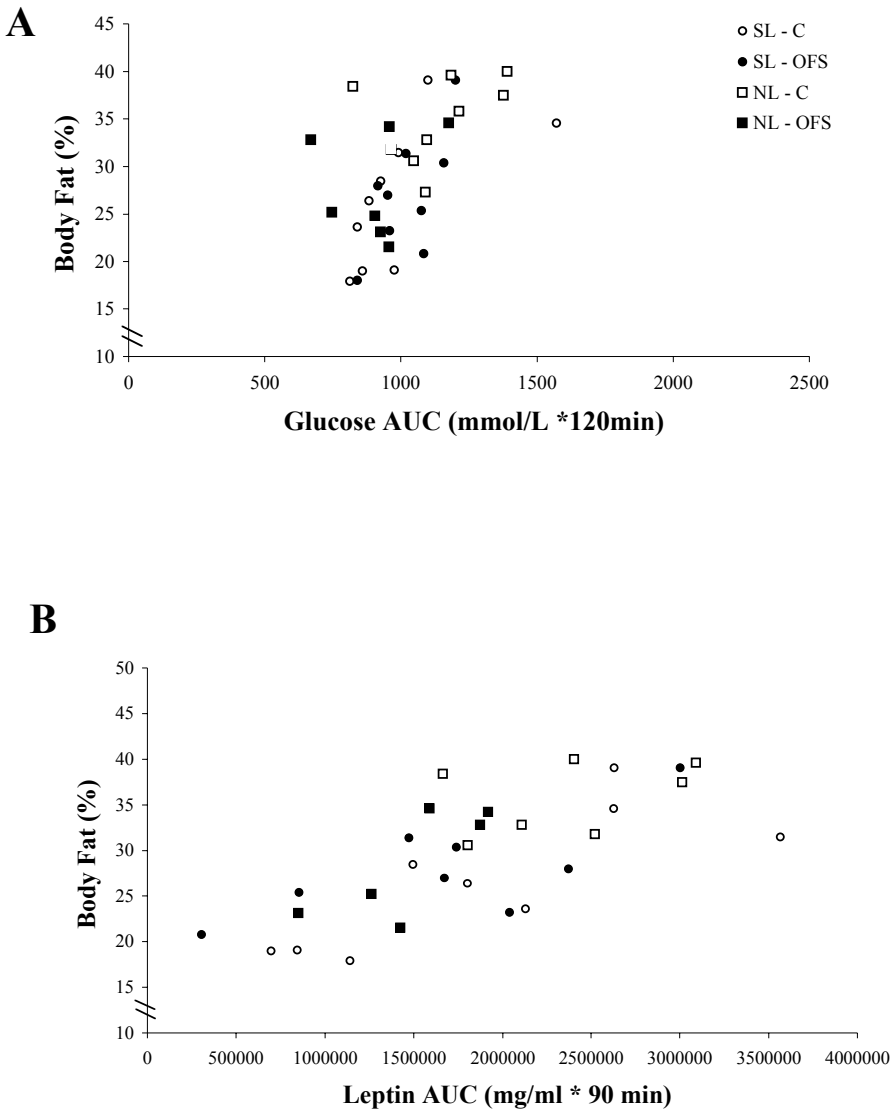

Figure B.1 Significant body fat (%) correlations with glucose and leptin measured during an OGTT in SL and NL rats weaned onto C or OFS diets. In panel A, $r^2 = 0.56$, $P<0.001$. In panel B, $r^2 = 0.72$, $P<0.001$.

Figure B.2 Body fat (%) correlations with fasting plasma leptin and ghrelin

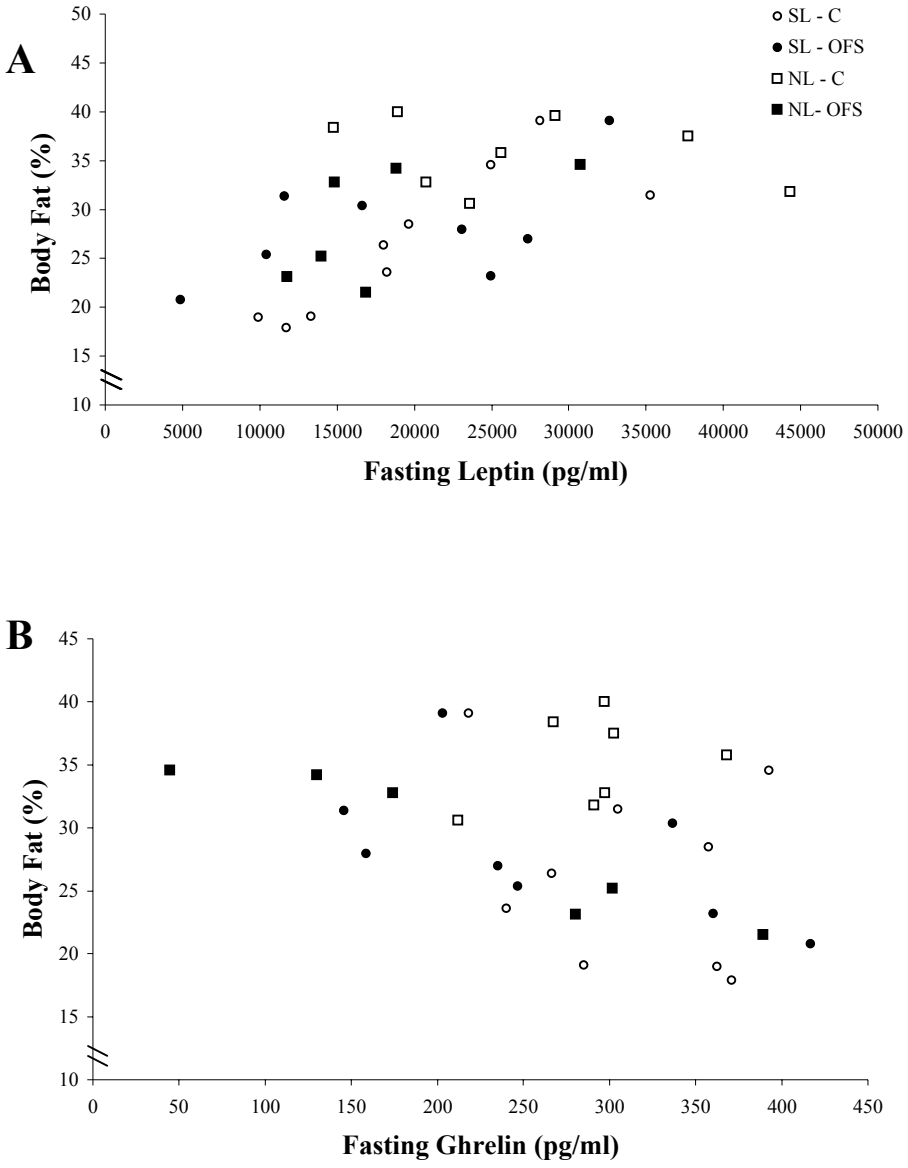

Figure B.2 Significant body fat (%) correlations with fasting plasma leptin and ghrelin measured during an OGTT for SL and NL rats weaned onto C or OFS diets. In panel A, $r^2 = 0.51$, $P<0.003$. In panel B, $r^2 = -0.34$, $P<0.05$.

Figure B.3 Body fat (%) correlations with glucose, leptin and GIP total AUC

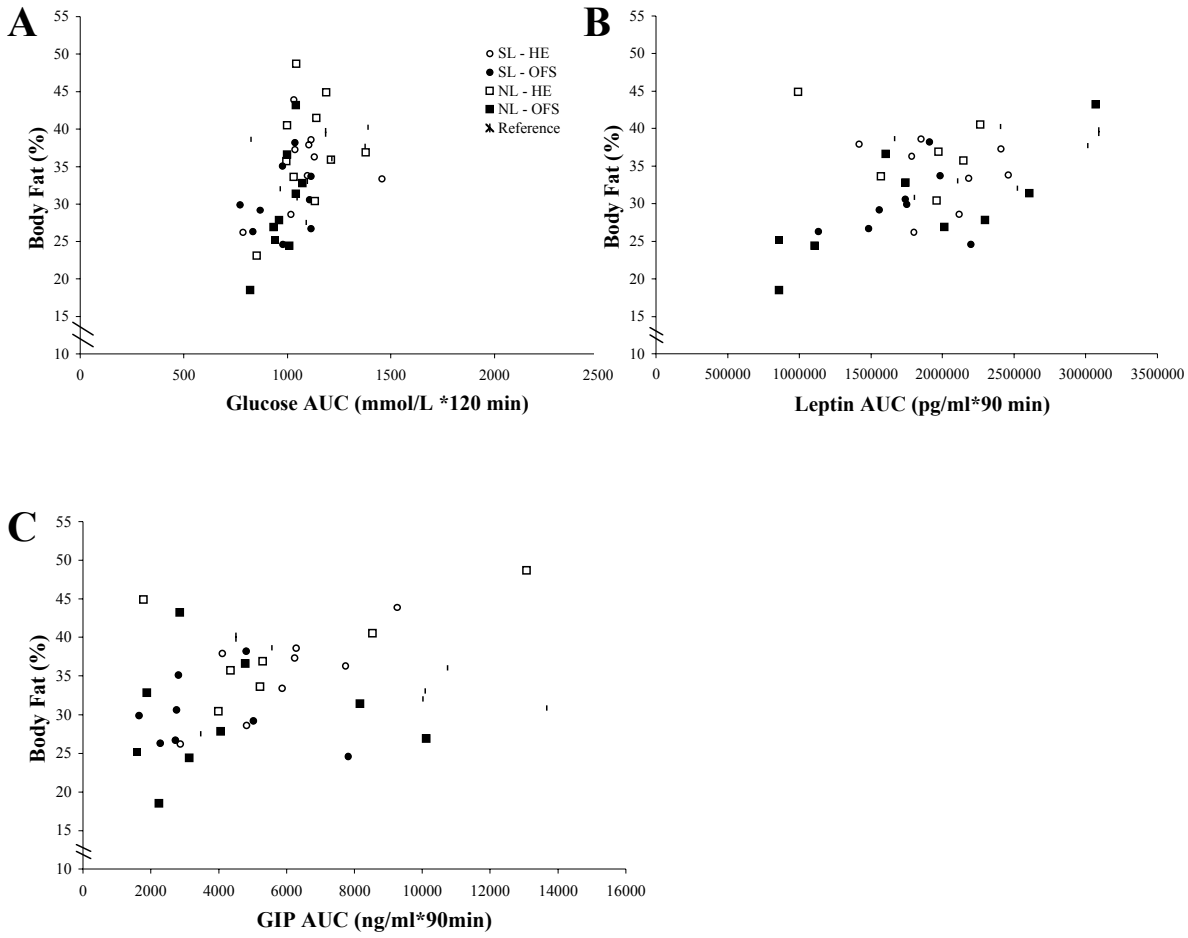

Figure B.3 Significant body fat (%) correlations with glucose, leptin and GIP total AUC measured during an OGTT in SL and NL rats weaned onto HE or OFS diets. In panel A, $r^2 = 0.53$, $P<0.001$. In panel B, $r^2 = 0.38$, $P<0.04$. In panel C, $r^2 = 0.41$, $P<0.02$.

Figure B.4 Body fat (%) correlations with fasting leptin

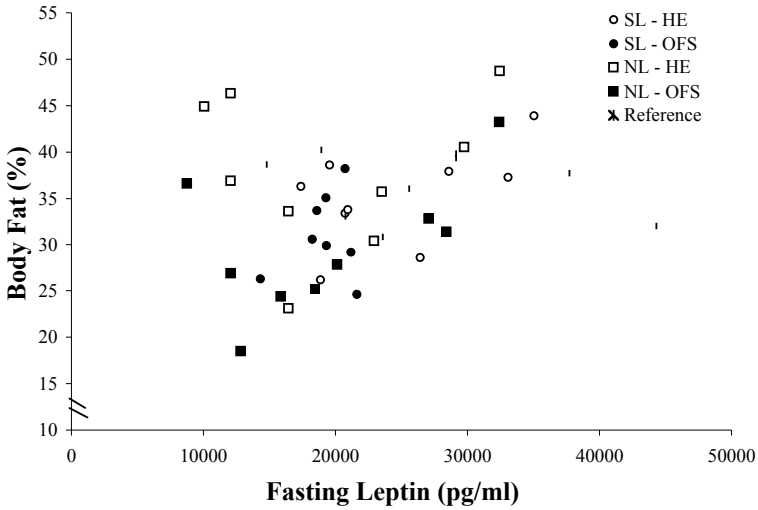

Figure B.4 Significant body fat (%) correlations with fasting leptin measured during an OGTT in SL and NL rats weaned onto HE or OFS diets ($r^2 = 0.34$, $P<0.04$).

APPENDIX C

Figure C.1 Absolute reliability for plasma insulin, amylin and leptin

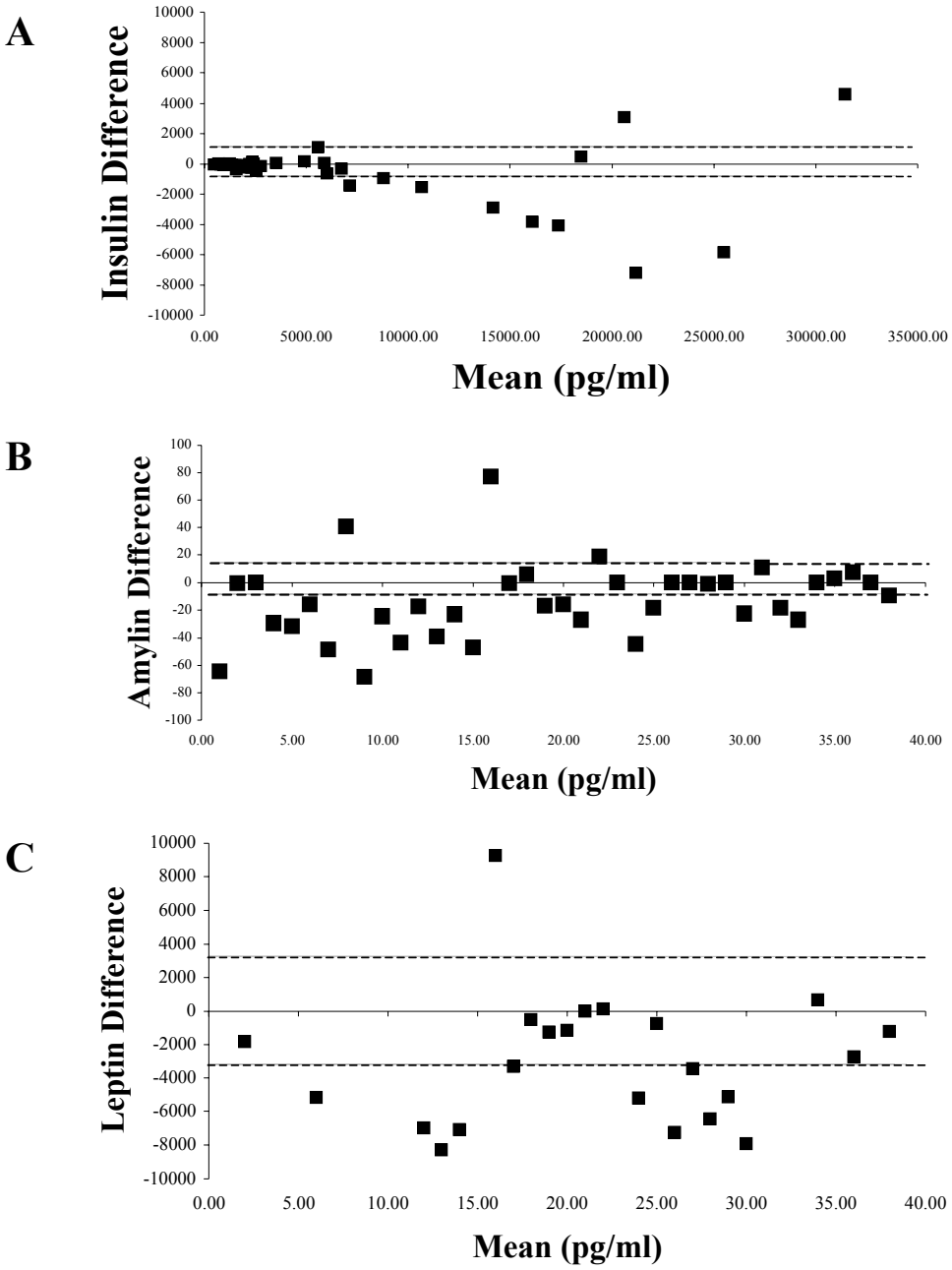

Figure C.1 Absolute reliability for plasma insulin, amylin and leptin using Milliplex assay. Dashed lines indicate 95% confidence interval.

Figure C.2 Absolute reliability for plasma PYY, GIP and ghrelin

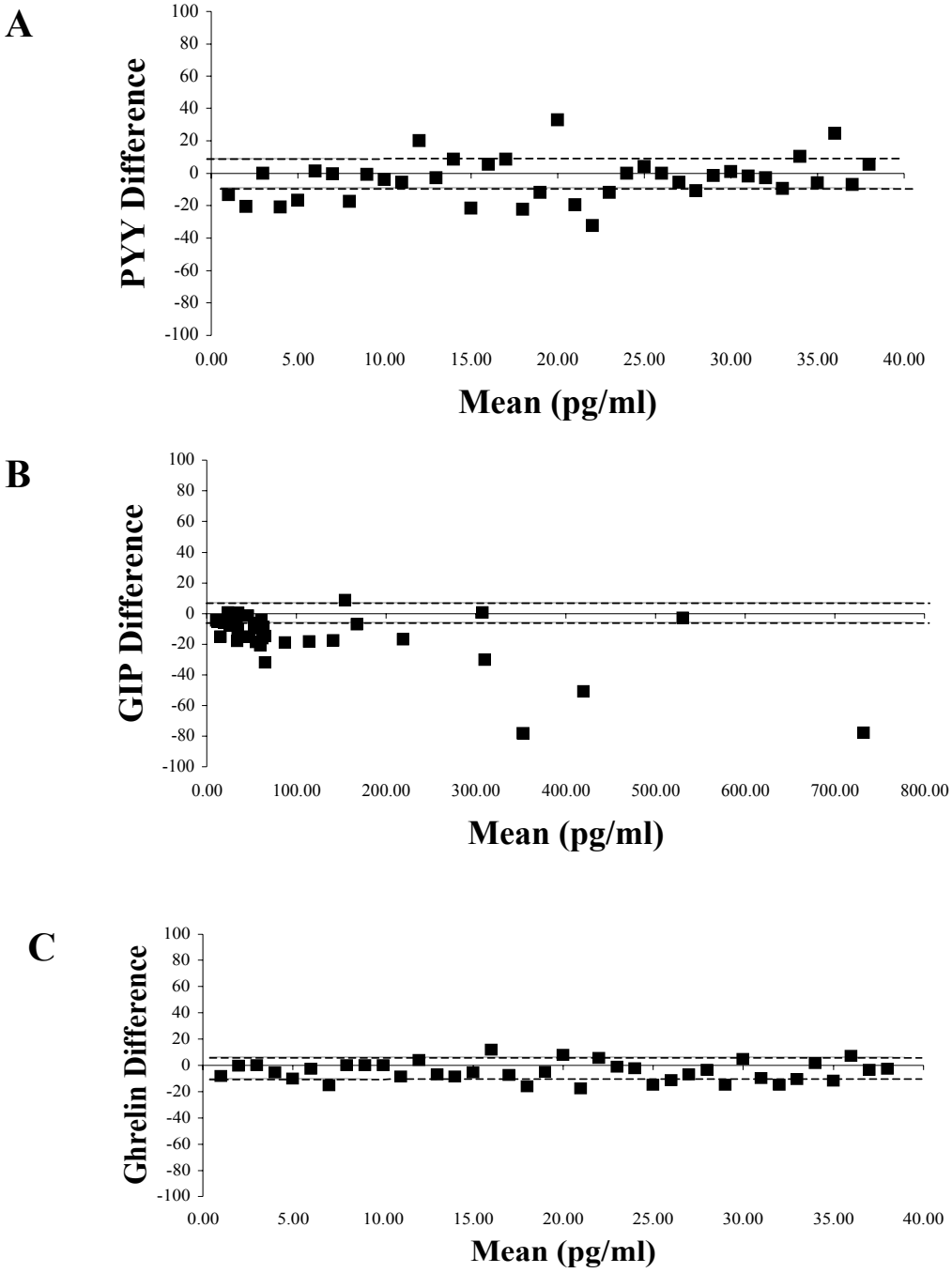

Figure C.2 Absolute reliability for plasma PYY, GIP and ghrelin using Milliplex assay. Dashed lines indicate 95% confidence interval.

Figure C.3 Absolute reliability for proglucagon and PYY expression in the ileum

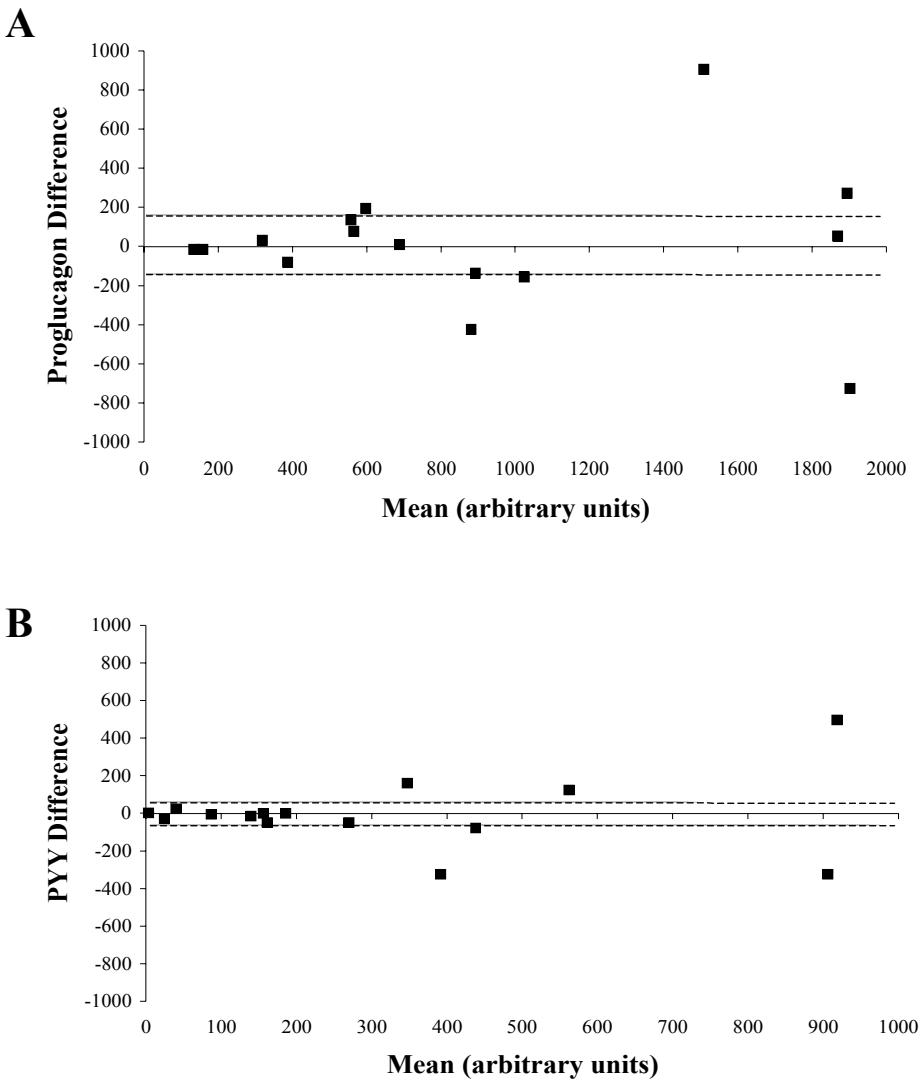

Figure C.3 Absolute reliability for proglucagon and PYY expression in the ileum using qRT-PCR. Dashed lines indicate 95% confidence interval.

Figure C.4 Absolute reliability for proglucagon and PYY expression in the colon

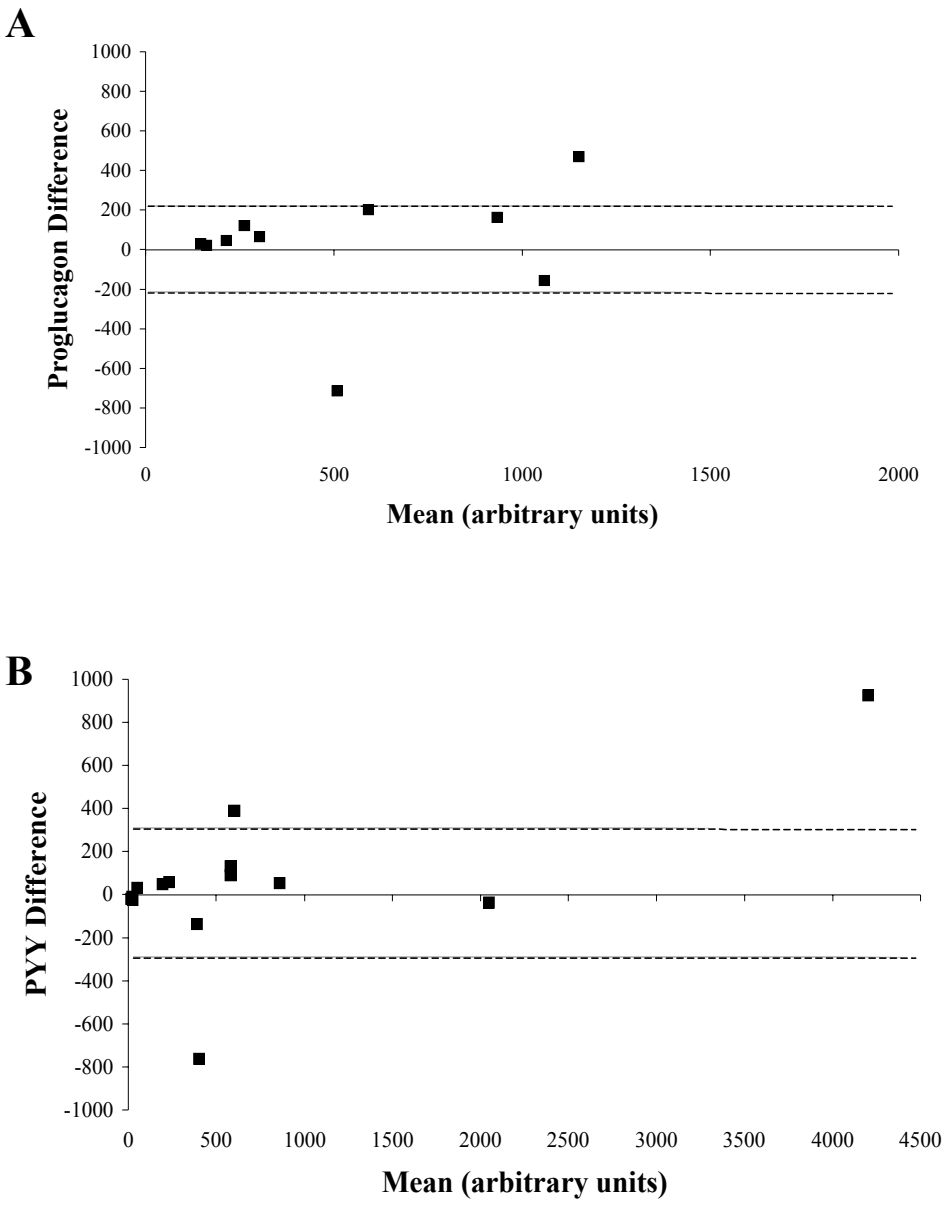

Figure C.4 Absolute reliability for proglucagon and PYY expression in the colon using qRT-PCR. Dashed lines indicate 95% confidence interval.

APPENDIX D

Table D.1 Quantitative rt-PCR primer sequences

Table D.1 Forward and reverse primer sequences used to assess gene expression in SL and NL rats.

Gene	Forward Primer	Reverse Primer
ACC	CCTTCTTCTACTGGCGACTGAG	TAAGCCTTCACTGTGCCTTCC
Beta-actin	TATCGGCAATGAGCGGTTCC	AGCACTGTGTTGGCATAGAGG
FAS	GAGGACTTGGGTGCCGATTAC	GCTGTGGATGATGTTGATGATAGAC
GAPDH	CAAGTTCAACGGCACAGTCAAG	ACATACTCAGCACCAGCATCAC
Proglucagon	ACCGCCCTGAGATTACTTTTCTG	AGTTCTCTTTCCAGGTTCACCAC
PYY	AGCGGTATGGGAAAAGAGAAGTC	ACCACTGGTCCACACCTTCTG
SREBP-1c	TCATCAACAACCAAGACAGTG	AGAGAAGCAGGAGAAGAGAAG