EFFECT OF FERMENTATION RATE OF DIETARY FIBRE ON SHORT-TERM SATIETY, LONG-TERM FOOD INTAKE AND GUT HORMONE RESPONSES IN MALE RATS

BY

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ABSTRACT

As obesity rates increased worldwide, nutritional strategies to reduce food intake as a weight management tool have gained much attention. Studies showed that dietary fibre can be protective against weight gain through fermentation that influences gut hormone levels to increase satiety and reduce food intake. Macronutrient-induced satiety to reduce meal size has also received a great interest. This study aimed to investigate the effects of macronutrients (carbohydrate, protein, fat) and fibres (different fermentation rates) on satiety, corresponding hormone responses (insulin, ghrelin, glucagon-like-peptide-1, peptide YY), and their relationships with food intake and body weight in rats. I found that diet containing fructooligosaccharides led to reduced long term food intake, weight gain and fat mass whereas wheat bran promoted food intake with unaffected weight gain. Both fructooligosaccharides and oil were associated with significantly lower food consumption and higher circulating PYY. My data suggested that satiety regulation is complex and can be strain-dependent.

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LIST OF ABBREVIATIONS

α-MSH	Alpha-melanocyte stimulating hormone
AgRP	Agouti-related peptide
ARC	Arcuate nucleus
CART	Cocaine- and amphetamine-regulated transcript
CCK	Cholecystokinin
cDNA	Complementary deoxyribonucleic acid
DF	Dietary Fibre
DPPIV	Dipeptidyl peptidase-4
F344	Fischer 344 rats
FOS	Fructo-oligosaccharides
GHSR	Growth hormone secretagogue receptor
GI	Gastrointestinal
GIP	Gastric inhibitory polypeptide
GLP-1	Glucagon-like peptide-1
GLP-1R	Glucagon-like peptide-1 receptor
GOS	Galacto-oligosaccharides
HPRT	Hypoxanthine phosphoribosyltransferase
ICV	Intracerebroventricular
LCT	Long chain triglyceride oil
MC4R	Melanocortin 4 receptor
MCT	Median chain triglyceride oil
mRNA	messenger RNA
NPY	Neuropeptide Y

PCR	Polymerase chain reaction
PGC	Proglucagon
POMC	Pro-opiomelanocortin
PYY	Peptide tyrosine tyrosine
RNA	Ribonucleic acid
RPL10A	Ribosomal protein 10A
RT	Reverse Transcription
SCFA	Short chain fatty acid
SD	Sprague-Dawley rat
WB	Wheat bran

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Chapter 1

Introduction

Introduction

The prevalence of overweight and obesity among humans is increasing both nationally and worldwide. It is a growing health concern as it increases the risk of health problems including hypertension, heart diseases, cancer and other chronic conditions. In 2005, obesity-related chronic conditions accounted for a $4.3 billion cost in Canada [1]. According to Statistics Canada, in 2011, approximately 40% of men and 27% of women were overweight and close to 20% of men and 17% of women were obese[2]. A study done in the USA showed that increased energy intake appears to the major driver of the obesity epidemic [3]. For humans, the desire to eat is not only driven by low blood glucose or other physiological indicators that prompt food intake for survival, but also by other factors including habit, opportunity, and/or social events. Thus, the amount of food consumed once the meal is initiated becomes a key physiological control to protect against weight gain. As a result, diet modifications as a weight management tool have gained great attention over the years. The mechanisms by which food regulates intake can be divided into those that influence short term food intake, which focus on prevention of overeating during the day, and long term maintenance that represents control over a longer period of time. Increasing evidence indicates that satiation (feeling of fullness) and short term satiety are mediated by a gradual buildup of gut hormones and hormonal signals released by the gastrointestinal (GI) tract in response to food ingestion. While insulin and ghrelin are the two hormones that are generally accepted to be associated with food intake, gut hormones such as peptide tyrosine tyrosine (PYY) and glucagon like peptide-1 (GLP-1) have been receiving a great deal of attention in the last decade. The regulation of these

hormones is complex; one of the important factors is the type of macronutrient since different macronutrients elicit hormonal secretions differently [4].

Insulin

Insulin is a peptide hormone with 51 amino acids and is secreted from the pancreas into the circulation where it acts as one of the key regulators of energy intake and utilization. Insulin secretion is proportionally stimulated by macronutrient intake. Insulin is best known for its role in mediating the homeostasis of circulating glucose. When there is excess circulating glucose, insulin stimulates an increase in glucose uptake by promoting glycogen synthesis, which increases glucose storage in the form of glycogen in the liver, muscle cells and adipose tissues. Other than carbohydrate metabolism, insulin also plays a central role in mediating fat metabolism. Insulin stimulates lipogenesis, promoting fatty acid synthesis from glucose and the subsequent production and storage of triglycerides in the adipose tissues. In parallel to that action, insulin also decreases gluconeogenesis and glycogenolysis in the liver to decrease glucose production. Conversely, when insulin is low, glucagon is secreted from the pancreas to trigger the release of glucose from liver by stimulating gluconeogenesis and glycogenolysis [5,6]. In general, insulin only rises after meal consumption because the postprandial rise in blood glucose is the main signal for insulin secretion. Insulin injections significantly reduce blood glucose [7]. The level of insulin decreases as blood glucose lowers and eventually falls back to baseline until the next food ingestion and this cycle continues [8].

Different macronutrients induce different levels of insulin release. It is generally accepted that, in humans, ingested carbohydrates such as glucose rapidly increase blood glucose, and subsequently elicit insulin release [9]. Although dietary fat tends not to elicit insulin release, it may suppress postprandial glucose levels by delaying carbohydrate absorption from the gut [10]. Protein elicits an insulin response similar to glucose but lower in magnitude. In additional, protein metabolism itself induces lower postprandial blood glucose than glucose. Proteins have been shown to elicit insulin release similar to carbohydrates but with lower postprandial blood glucose levels [9-11]. For example, adding whey proteins to a carbohydrate meal has been shown to bring about noticeably reduced postprandial blood glucose levels and higher total insulin and peak insulin secretions than carbohydrates alone in humans [12-14]. It is suggested that whey proteins are potent insulin secretagogues because they have a high content of five amino acids: isoleucine, leucine, valine, lysine and threonine, which have been shown to be highly insulinogenic [12,15].

Rat studies have shown similar results to those in human subjects. A duodenal infusion of isocaloric nutrients showed that the insulin peak at 30 minutes was increased in the order of glucose > protein >> fat, where protein elicited much lower blood glucose than glucose, and fats had virtually no effect on blood glucose. The effect was short-lived and insulin levels were not different among the nutrients after 90 minutes[16]. Glucose gavage to the stomach also increased blood glucose whereas corn oil had no effect[17]. Taken together, the insulin response to nutrients is generally the same between rats [16] and humans[9,18].

Ghrelin

Posttranslational modification of the ghrelin precursor produces active ghrelin with an octanoylation on the first serine residue. Active ghrelin, containing 28 amino acids, is a gastrointestinal peptide and an endogenous ligand for the growth hormone secretagogue receptor (GHSR) in the hypothalamus. Active ghrelin is the only form of ghrelin that binds to the GHSR [19]. Ghrelin is highly conserved evolutionarily in mammals so it is very similar in humans and rats, differing by only two amino acids. Ghrelin is mainly produced by the endocrine cells of the mucosa in the stomach fundus. In addition to stimulating growth hormone secretion, ghrelin is a strong mediator of satiety. Ghrelin promotes food intake, body weight gain and adiposity by acting through GHSR. Administration of ghrelin significantly increased both growth hormone secretion and food intake in wild-type rodents whereas both GH and food intake were unaffected in GHSR-null rodents [20,21]. While there are numerous gastrointestinal hormones that are associated with reduced food intake, ghrelin is the only known orexigenic hormone to stimulate food intake in a calorie dependent fashion. Conversely, ghrelin secretion is also suppressed by food intake. Therefore, ghrelin is often recognized as a hunger indicator since circulating ghrelin increases in the fasted state, rising up to two-fold prior to a meal and falling progressively after food intake in both humans[22-24] and rats [16,25,26]. Ghrelin has been implicated in meal initiation and adiposity promotion. Ghrelin receptor antagonists decrease food intake in rats [27]. The peptide has been associated with obesity: obese rats had significantly higher basal levels of ghrelin and higher food intake compared to lean rats [27,28]. Furthermore, ghrelin directly and acutely promote food intake and is rapidly cleared from the blood. For example, food intake was increased after ghrelin

administration but only up to one hour post administration. A second administration four hours later also led to similar results [29]. Chronic administration led to higher cumulative food intake and excess weight gained in rats, since the effect is not easily saturable [26]. The route of administration does not seem to play a role in the ghrelin effect. Many different types of exogenous ghrelin administration, such as intraperitoneal (i.p.), intravenous (i.v.) and intracerebroventricular (i.c.v.) injections have all been shown to increase food intake. Ghrelin also increases food consumption in a dose dependent manner [26,29-31], reduces spontaneous locomoter activity, and reduces fat utilization in rats [30-32]. These findings support the role of ghrelin in regulating long term energy homeostasis and short term food intake.

Although ghrelin is suppressed following food intake, macronutrients do not suppress ghrelin equally. Glucose and protein suppress ghrelin to a similar extent and these are much stronger suppressants than fat. All three nutrient deliveries (gavage to stomach, infusion into the duodenum or jejunum) showed ghrelin suppression in rats [16,25,31]. In healthy men, postprandial ghrelin is suppressed after eating a meal mixed with all three types of macronutrients and this effect is also calorie dependent. Both protein and carbohydrate have been shown to suppress ghrelin but fat is considerably poorer at suppressing ghrelin in humans [18,23,33].

The interaction between ghrelin and food intake is complex because it affects other hormones. There is an inverse relationship between ghrelin levels and circulating insulin and it appears that insulin inhibits ghrelin secretion and vice-versa [34]. Thus, feeding nutrient preloads results in opposite levels of ghrelin and insulin [16] (ie. a higher insulin release correlates with greater ghrelin suppression) and the ghrelin response in a

fasted state is inhibited by insulin injection[25]. Obese individuals have reduced basal ghrelin levels and ghrelin levels do not respond to food intake. The level of ghrelin remains fairly constant in obese men while it falls rapidly in healthy men after the same meal[23,33]. While the level of fasting ghrelin is negatively associated with insulin, it is positively associated with insulin sensitivity [35]. Therefore, high circulating ghrelin is associated with lower insulin resistance [36]. This is also true for obese individuals that are insulin resistant; they have significantly lower fasted ghrelin levels than those that are insulin sensitive [37]. It is possible that greater calorie content is necessary to elicit a ghrelin response in obese subjects. A failure to respond to food intake may be due to both the loss of ghrelin and insulin sensitivity. As a result, this can lead to overconsumption of calories and obesity. The sedentary behaviour may be another reason for increased prevalence of obesity.

Glucagon-like peptide-1 (GLP-1)

The proglucagon gene is expressed in the hypothalamus, pancreas and distal GI tract. The proglucagon gene encodes several peptide hormones, all derived from the original proglucagon peptide but that undergo tissue specific posttranslational modifications. GLP-1 and GLP-2 are the two peptides that are derived from the proglucagon gene in the lower GI tract and they are abundantly produced by the enteroendocrine L cells[38]. Both GLP-1 and GLP-2 are 50% homologous to glucagon, but only GLP-1is an incretin. Incretins are circulating hormones secreted from the GI tract to enhance insulin secretion following food intake, thereby reducing blood glucose levels.

GLP-1 is one of the two incretin hormones discovered [39]. There are two isoforms of GLP-1, GLP-1 (7-37) and GLP-1 (7-36) amide[40]. Both forms have insulinotropic activity in humans [41,42] and rats [43,44]. Food intake is the primary proglucagon gene expression regulator in the lower GI tract [45]. GLP-1 has a half-life of approximately 2 minutes due to rapid degradation by dipeptidyl peptidase-4 (DPPIV). DDPIV processes several protein hormones *in vivo*, cleaving peptides from the N-terminal of proteins with a proline or alanine in the second position. There is no requirement for specificity in the N-terminal amino acid [46]. More than half of the active GLP-1 is degraded into inactive forms, GLP-1 (9-37) and GLP-1 (9-36) amide, before entering the circulation [39].

Because GLP-1 is secreted from the enteroendocrine cells in the gut, hormonal production is mediated by nutrient intake. Active GLP-1 is rapidly secreted within minutes post-meal, and in general, GLP-1 is noticeably higher around 30 minutes after ingestion has started [47,48]. Since GLP-1 is regulated by nutrients, the duration of elevated GLP-1 levels depends on the meal size and macronutrient composition [49]. The nutrient-generated signals to release GLP-1 are transmitted to the endocrine cells in the lower gut by both neural control and directly by nutrient contact. As GLP-1 is rapidly released from the lower GI tract, the initial GLP-1 release cannot be mediated by direct nutrient contact [22]. The effect of nutrient transport along the GI tract gives rise to the phenomenon where two postprandial active GLP-1 peaks are observed, one at 30 minutes post-ingestion followed by a slight fall, and then another rise at 60 minutes [49,50]. Like GLP-1, gastric inhibitory polypeptide (GIP), that is most abundant in duodenum and jejunum of the GI tract, is another incretin that enhances insulin releases in response to blood glucose. GIP has also been implicated in promoting the production of proglucagon-derived peptides.

Therefore, upon food ingestion, GIP may serve as a neural regulator that initiates GLP-1 secretions [51].

Intracerebroventricular, intraperitoneal, and intravenous administration of active GLP-1 significantly reduced food intake in a dose-dependent manner in rats [52-54]. Mixed results are often observed in human studies due to differences in the duration and rate of GLP-1 infusion and subject characteristics. Nevertheless, a meta-analysis of nine human studies done on the effect of GLP-1 infusion on energy intake showed that GLP-1 administration decreased subsequent energy intake but its effect was similar in lean and obese subjects [55].

As an incretin hormone, the primary stimulus of active GLP-1 is nutrient intake. However, the three types of macronutrients influence active GLP-1 secretion differently, as will be further discussed later. An important role of active GLP-1 is to enhance glucose-dependent insulin release [40], and in accordance with this role, glucose is an effective GLP-1 secretagogue and directly increases insulin secretion in the presence of circulating glucose. GLP-1 enhances insulin release during a glucose challenge in both healthy and type II diabetic patients. However, GLP-1 mediated insulin secretion only occurs in the presence of blood glucose [56,57].

Jejunal, duodenal and ileal infusion of glucose have all been shown to significantly increase active GLP-1 release. In addition, ileal and duodenal infusion of glucose increased active GLP-1 secretion in a dose-dependent manner [48,58]. An interesting study investigated the sensitivity of GLP-1 release in response to different types of carbohydrates that were luminally perfused into the ileum. Glucose induced significantly

higher levels of GLP-1 after 10 minutes, but GLP-1 returned to baseline after 30 minutes. In contrast, fructose and galactose increased GLP-1 more gradually and the levels remained elevated at 30 minutes [59]. This suggests that carbohydrate is a potent stimulant of GLP-1 secretion, but the effectiveness can vary among carbohydrate types.

Fats are also potent stimulators of active GLP-1 release. Both jejunal infusion of linoleic acid and duodenal infusion of oil have been shown to significantly increase active GLP-1 dose dependently [48,58,60,61]. Moreover, fatty acid was found to induce GLP-1 secretion in rat intestinal cell cultures, which suggests that fat itself may be acting at the cellular level [62].

Unlike glucose and fat, protein does not appear to influence active GLP-1 release. A duodenal protein infusion at a dose as high as one third of the daily intake produced GLP-1 secretions that were not different from the control [48]. Jejunal infusion of casein also showed no effect [58]. Collectively, since direct nutrient delivery to the GI tract indicates whether a link between nutrient and GLP-1 production exists, protein intake does not appear to enhance GLP-1 production.

Peptide YY (PYY)

Peptide YY was first isolated from porcine small intestine. The straight chain polypeptide of 36 amino acids has tyrosine residues at both the carboxyl and amino terminals of the peptide [63]. Like GLP-1, PYY is produced by the enteroendocrine L cells in the lower GI tract, and the localization close to the lumen suggests that luminal content may play a role in PYY release. PYY is found in both rats [64-66] and humans [38,67]. Its

distribution is fairly similar in mammalian species, such as human, dogs and rats, along the GI tract where PYY increases distally starting with low to non-existent amounts in the stomach. The highest levels of PYY are found from ileum to the colon; the colon has up to 100 fold more PYY immunoreactivity than duodenum [64,65,68,69].

Circulating PYY has a half-life of about 8 minutes [70] and is one of the DPPIV targeted substrates. PYY exists in two active forms of PYY (1-36) and PYY (3-36), where the latter is truncated by DDPIV at the tyrosine-proline residues on the N terminal end [46, 71,72]. PYY belongs to the pancreatic polypeptide family along with NPY. PYY shares structural homology with NPY with 24 identical amino acids and acts by binding NPY receptors [71,72]. PYY (1-36) is able to bind and activate receptors Y1, Y2, Y4 and Y5. However, PYY (3-36) is more selective for Y2 receptors due to the removal of tyrosine and proline at the N terminus. The Y2 receptors are important in mediating satiety as shown by abolition of the anorexic effect of PYY in the presence of a Y2 receptor antagonist. Binding of PYY (3-36) to the Y2 receptors also inhibits NPY release, which will be further discussed later [72-75]. The proportion of these two active forms reportedly differs according to feeding status. A study found that the two active forms exist in approximately equal amounts in fetal intestinal tissues [76] while another study indicated that PYY (3-36) accounts for 37% of total basal PYY and increased up to 54% postprandially [77]. PYY (3-36) is suggested to be 10-fold more potent than PYY (1-36) in terms of food intake inhibition [78]. PYY (3-36) is more potent in food suppression than PYY (1-36) may be due to that PYY is rapidly cleaved to PYY (3-36), which has a high affinity for Y2 receptor. On the other hand, while PYY (1-36) binds to the Y2 receptor, it also causes overeating in rats through binding to the Y1 receptor [71]. The effects of

uncleaved PYY protein on food intake suppression are saturated by the balance between the binding to Y1 and Y2 receptors. Not much is known about the regulation of the DPPIV mediated protein cleavages. However, while DDPIV rapidly inactivates GLP-1, it alters the biological activity of PYY into a selective agonist at the Y2 receptor.

There is a clear relationship between PYY and food intake. Administration of exogenous PYY by intraperitoneal and intravenous routes as well as via pulmonary delivery have been shown to decrease food intake in both rats [53,78,79] and humans[72,80,81]. Injection of PYY (3-36) into the ARC prior to the onset of dark cycle significantly decreased subsequent food intake in both free feeding rats and rats previously fasted for 24 hours [79]. Exogenous PYY has been shown to inhibit food intake up to 12 hours in both rodents and men [79]. PYY (3-36) infusion into rats showed that length of infusion time also plays an important role in cumulative food inhibition. The same amount of PYY (3-36) delivered at a higher rate for 15 minutes suppressed food intake for up to 3 hours whereas when delivered over a 3 hour period showed cumulative food intake reduction for 11 hours [78]. In addition, continuous PYY (3-36) administrations for 7 days significantly reduced accumulated food intake and body weight gain [79].

Circulating PYY is increased following food intake in rats and becomes significantly higher than the basal level within 30 to 60 minutes following food intake [47,69,82]. Similar to GLP-1, PYY is rapidly released following food intake [82], and this action occurs before the nutrients reach the L cells in the lower GI tract, where PYY is mainly produced. The gut hormone cholecystokinin (CCK) is released in the upper GI tract following food intake and has been shown to induce PYY secretions. Therefore, upon

nutrient ingestion, CCK release may serve as a foregut signal to initiate PYY release from the lower GI tract [83].

Rat studies have shown that different types of macronutrients affect PYY release differently. Jejunal infusion of linoeic acid gave the highest increase in PYY, followed by casein. Oppositely, jejunal infusion of glucose decreased total PYY [58]. Glucose infused into the ileum or colon at physiological concentrations did not influence PYY secretion but significantly increased PYY levels when presented at a supraphysiological concentrations [84,85]. Although a protein hydrolysate (peptone) infused into the ileum elicited PYY release [84], a mixture of amino acids as a form of protein infused into the colon was able to induce PYY release only at a much higher than normal physiological concentration [85]. Therefore, the specific type of macronutrient or region of infusion may contribute to the variation in PYY release. A regional effect was again apparent when a supraphysiological concentration of oleic acid infused into the colon had no effect on PYY release[85] whereas a significant effect at a much lower concentration was observed when it was delivered straight to the duodenum or ileum[69]. Moreover, duodenal infusion significantly increases PYY secretion after 120 minutes when ileal infusion only required 60 minutes. This further supports the idea that direct nutrient stimulation in the lower GI tract plays a vital role in mediating PYY secretion[69].

Arcuate nucleus

The arcuate nucleus (ARC) of the hypothalamus is a crucial site for control of appetite and feeding in the central nervous system; it receives and integrates signals of nutritional status. Multiple hormones, including the ones discussed earlier, converge in the ARC to regulate food intake (Fig. 1.1). There are two distinct types of neuronal populations that have important but opposite roles in energy homeostasis regulation. The neuronal activities in the ARC are often classified as "first order neurons" of the hypothalamic response to hormonal signals. Those neurons can project to the "second order" neuropeptide neurons located in other hypothalamic sites such as the paraventricular nucleus (PVN)[86]. Inhibition of the neurons in the ARC affects peptide production and activation of these neurons increases mRNA expression and protein production. Peptides produced in the ARC are released to the other parts of hypothalamus such as the lateral hypothalamus (LHA) and the PVN, where they have active roles in appetite regulation [86]. The neurons serve as the primary signal sensor for food intake from the gut and they generate a series of downstream cascade responses [70]. Therefore, the balance of activity between these neurons in the ARC has a great impact on food intake.

The orexigenic neurons, neuropeptide Y (NPY) and agouti-related protein (AgRP), stimulate food intake. NPY neurons are predominately localized in the ARC and are able to project centrally to the other parts of the hypothalamus such as the LHA and PVN. AgRP neurons are mostly co-localized with NPY neurons; AgRP is exclusively synthesized in the ARC. Likewise, the anorexigenic neurons project to many different sites within the hypothalamus. They contain pro-opiomelanocortin (POMC), and cocaine- and amphetamine-regulated transcript (CART), which suppress food intake [87].

Both NPY mRNA expression in the ARC and amount of NPY released to the PVN are significantly higher in food deprived rats[88]. Intracerebroventricular injection or administration of NPY into the PVN significantly stimulates food intake [29,89]. Intracerebroventricular injection of AgRP has also been shown to promote food intake in rats [29,32,52,90]. The orexigenic effect of NPY lasted approximately 8 hours post-injection, while rats receiving AgRP showed significantly higher food intake up to 72 hours post injection. Locomoter activity was unaffected by NPY but significantly suppressed by AgRP administration [32]. NPY mRNA expression was significantly higher in 4 hour fasted rats but the effect was transient and decreased after this time whereas AgRP remained elevated at 24 hours in fasted rats [91]. Those results showed that the expression of both NPY and AgRP are relatively long-lived. Although NPY is generally accepted as being the most orexigenic molecule, its effect is relatively shorter-lived compared to AgRP [86].

The other distinct neuron populations with anorexic property include POMC and CART neurons. POMC neurons are concentrated mostly in the ARC; therefore, most of the POMC peptides are produced in the ARC. The POMC gene encodes a protein of 285 amino acids of which 44 are removed during translation to produce the POMC peptide. The POMC peptide has eight potential cleavage sites, so POMC is a precursor that can undergo extensive tissue specific posttranslational processing to yield up to ten biologically active peptides. POMC is first cleaved into either pro-adrenocorticotropic hormone (ACTH) or β-lipotropin, which are further cleaved into other peptides including β-endorphin and α-, β - and Ɣ-melanocyte stimulating hormones [92,93]. Those POMC-derived peptides, mostly act through the melanocortin pathway via activation of melanocortin receptors, have established associations in various biological functions such

as skin pigmentations, adrenal steroidogenesis, and energy balance [92-94]. The α-melanocyte stimulating hormone (α-MSH), is the main energy regulator of this family acting through the melanocortin pathway via the melanocortin 4 receptors (MC4R) [34,91] to decrease food intake in rats[90,95,96]. Intracerebroventricular administration of α-MSH significantly reduced food intake while the same amount of AgRP injection completely blocked the anorexic effect of α-MSH by acting as a MC4-R antagonist. Therefore, neuron activation of one population not only increases the subsequent protein production, but often also inhibits the neuron activity of the opposite neuron population [90]. Similarly, intracerebroventricular injection of CART significantly reduced food intake in rats [90].

Rat studies have shown a clear link between the neuropeptides and food intake. Fasted rats showed higher NPY and AgRP mRNA expression as well as reduced POMC and CART mRNA expression. The POMC and CART mRNA levels were normalized in fasted rats after re-feeding [97,98]. The alternation of neuropeptide expressions according to the feeding indicates that feeding-related signals exert a strong impact on the level of peptides present in the ARC. There is evidence suggesting that the action of hormones on satiety control, at least in part, is via their effects on neural activity in the ARC, which has a great impact on the neuropeptide peptide production. The balance of the neural activity is regulated by hormonal signals from adipose tissue (leptin, insulin), or other tissues (insulin) as well as GI tract hormones (ghrelin, CCK, GLP-1, and PYY) [91,99].

In addition to acting on the gut and peripheral tissues, a portion of circulating insulin crosses the blood brain barrier where it binds the insulin receptor, a transmembrane tyrosine kinase receptor that is the only target receptor for insulin in the ARC. Because insulin receptors are abundantly expressed in the ARC, insulin can

influence neuropeptide expression there. In an insulin deficient state, such as occurs in Type I diabetics or during fasting, NPY and AgRP neurons are activated and POMC and CART neurons are inhibited [88,91]. An intracerebroventricular administration of insulin or an insulin analog led to reduced food intake in a dose dependent manner, significantly increased POMC expression and suppressed NPY expression in rats [100,101].

The gut hormones GLP-1 and PYY have also been shown to affect ARC neuron populations in a fashion similar to insulin [102]. Intracerebroventricular administration of GLP-1 affected the neuropeptide expression pattern associated with a fasted state and resulted in significantly reduced NPY / AgRP mRNA levels and higher POMC / CART mRNA levels [98]. A GLP-1 receptor (GLP-1R) is expressed in several locations including the hypothalamus and lower gut. Intracerebroventricular administration of GLP-1 reduced food intake in fasted rats [98] and continuing the treatment for six days led to weight reduction[52]. On the other hand, administration of the GLP-1R antagonist (exendin 9-39) increased food intake and led to weight gain after 3 days. Exendin 9-39 and NPY administration together promoted food intake and weight gain in an additive fashion [52] implying that GLP-1 acts through GLP-1R to mediate food regulation and the receptor antagonist can interact with NPY to further enhance food intake.

Both injection of PYY into the ARC and PYY pulmonary delivery were associated with a reduced NPY neuron activity, an increased POMC neuron activity and corresponding changes in mRNA expression in the ARC. The NPY activity inhibition subsequently increased POMC neuron activity [79,103,104]. Furthermore, a single injection of PYY (3-36) directly into the ARC also reduced food intake in fasted rodents. However, the effect was not seen in Y2 receptor null rodents; while the rodents responded to PYY

(3-36) dose dependently in terms of food intake reduction, an anorectic effect was absent. Altogether, the data suggest that PYY may increase satiety by directly acting through the Y2 receptor in ARC to increase the anorexigenic activity of POMC while suppressing the orexigenic activity of NPY neurons [79]. Ghrelin is the only gut hormone with an orexigenic effect. Like the hormones above, ghrelin also acts through ARC, which is the hypothalamic site that is most responsive to ghrelin injection in terms of stimulating food intake [29,105]. Ghrelin has been shown to activate NPY and AgRP neurons in the ARC and intracerebroventricular injection of ghrelin increased NPY and AgRP mRNA expression in the ARC [106-108]. Taken together, it is apparent that gut-level hormones that mediate food intake have a great impact on the activation and inhibition of the ARC neuropeptides. Therefore, the hormones mediate satiety at least partially by acting through ARC to influence the output of the neurons.

Dietary Fibre

The potential beneficial role of dietary fiber (DF) has also received a great deal of attention. One of the many health benefits claimed in the scientific literature links DF with increased short term satiety and decreased long term risk of weight gain.

Different jurisdictions have slightly different definition for DF. In Canada, DF is defined as

> *"Dietary fibre consists of the endogenous components of plant material in the diet which is resistant to digestion by enzymes produced by humans. They are predominantly non-starch polysaccharides and lignin and may include, in addition, associated substances"* [109].

Despite differences in DF definitions, the literature has linked DF consumption to lower food intake over the short term and less weight gained over the long term. The majority of controlled studies have shown that consumption of DF, including soluble, insoluble and mixed fibres, is positively associated with increased satiety over the short term [110,111]. Over the long term, refined grains were more likely to be associated with more weight gained while cereal and whole grain intakes have been correlated to lower weight gain [112,113]. Observational studies over 6, 8 and 12 years showed that higher DF intake was associated with lower weight gain, suggesting that DF has a role in protecting against weight gain [112-114].

Even though DF is made of carbohydrates, it is not digested by the GI tract and passes into the large intestine, where it can be fermented by the resident microflora to produce short chain fatty acids (SCFA). SCFA are thought to influence satiety via changes in gut hormone release. Although not digested in the stomach or small intestine, several proposed roles in the upper GI tract have been suggested for DF, including attuning the rate of stomach emptying, alterations in the motility of digesta along the small intestine, and slowing the rate of digesta absorption. All these factors combine to alter the oral-fecal transit time and are suggested to contribute to short term satiety by increasing the feeling of fullness [115].

DF is thought to act via promotion of gut hormone release and signaling. As mentioned earlier, PYY and GLP-1 are the two gut hormones that are produced in the lower GI tract, which is the site of DF fermentation. An increase in gut hormone levels is linked to reduction in both food intake and body weight in rats [116,117]and in humans [118].

The proposal that this is a primary mechanism for control of food intake comes from studies showing that DF intake increased PYY and GLP-1 secretion in both rats [47,119] and humans [118]. The DPPIV protease that rapidly influences the activity of PYY and GLP-1 is unaffected by diet with or without additional DF [119], which suggests that gut hormones levels are directly modulated through diet.

Dietary fibre can be classified using several different criteria. There are polymers that are soluble in hot water (soluble dietary fibre) and polymers that are insoluble in hot water and detergent (neutral detergent dietary fibre or insoluble dietary fibre). These designations were originally thought to be related to fermentability in the lower gut but this has been disproven and they are now largely ignored. It has been recommended that the term fibre solubility should be replaced by the specific physiological effects of DF, such as fermentability or viscosity. Some polymers (mostly beta-glucans from oat and barley) increase the viscosity of the small intestine and have been shown to prolong transit time and absorption of nutrient [120]. DF from foods naturally high in DF is usually mixtures of polymers with some protein attached and primarily represents plant cell wall material (wheat DF, oat DF). Simpler forms of fermentable material are usually associated with manufactured polymers such as fructooligosaccharides (FOS) or galactooligosaccharides (GOS) or highly purified material from plants (e.g. resistant starch). Differences in the complexity and water solubility of the carbohydrate polymers, as well as association with lignin and protein can influence the rate of fermentation and potentially lead to alterations in gut level hormone responses. FOS and wheat bran (WB) represent two materials at different ends of the fermentation rate spectrum. Naturally occurring fructans [fructose polymers linked by $\beta(2\rightarrow1)$ bonds with a terminal $\alpha(1\rightarrow2)$

linked D-glucose] are found as inulin; this long chain carbohydrate (degree polymerization of 20-30 with some branching) is isolated primarily from chicory and Jerusalem artichoke but also found in onions, leeks and even wheat bran. The man-made version is called FOS and is usually much shorter (degree polymerization of 4-12) and highly branched [121]. These simple carbohydrates are very soluble in water and are fermented at a much faster rate than WB. For example, 99% of the inulins are fermented by 4 hours whereas only approximately 41% of the WB is fermented in 24 hours [122]. FOS has a shorter chain and therefore is fermented more rapidly than inulin [121]. FOS is considered to be soluble fibre because of its ability to form a gel-like material when in contact with water [123]. On the other hand, WB is a source of insoluble fibre, and does not readily absorb or dissolve in water [124]. It is composed of several different polymers including arabinoxylans, fructans, hemicelluloses and cellulose. It is highly lignified, giving the polymers strength in the cell wall but resulting in closed structures that are not quickly fermented. Due to the different fermentability properties of FOS and WB, the diets used in the studies described in this thesis were adjusted so that the total amount of fermentation between the two types of fibers was constant. It is thought that the slower fermentation of WB produces lower peaks of SCFA but sustained SCFA production, unlike FOS, therefore yields more SCFA [122].

As discussed earlier, both injection and infusion as a delivery method have shown a clear relationship between hormone levels and food intake. However, these direct delivery methods could provide a much higher than normal concentration of nutrients or hormones. This would let experimenters better identify the links among hormones secretions, nutrient exposures and food intake but those paradigms do not mimic the

normal physiological conditions even though infusion of gut hormones is often meant to mimic normal postprandial levels. There is strong evidence showing a correlation between food intake and fibre intake, food intake and hormonal secretions, as well as fibre intake and hormonal secretions. However, studies lack a full examination of the relationship among DF intake, hormone levels, and food intake, and the role that macronutrients play in this process. While long term observational studies showed that fibre intake was positively associated with reduced weight gain, these studies provided no information on the influence of the type of DF on food intake. It is hoped that a more detailed understanding of the mechanisms regulating satiety will lead to the development of novel therapeutic strategies to suppress food intake and ultimately decreases risks of obesity.

Aims and Hypotheses

The main aim of the studies described in this thesis is to investigate the relationships among fermentation, gut hormone secretions, macronutrients and food intake. Chronic exposure to DF (FOS and WB) was used to investigate the effect of fermentation on long term food intake and basal hormone levels in order to determine whether the rate of fermentation influences basal hormonal secretions that could lead to reductions in food intake and weight gain. This study also examined the effects of the three types of macronutrients on hormonal secretions. Because effects of macronutrients on food intake has been widely examined, I aimed to reproduce the effect of macronutrients in order to further investigate their links to hormonal responses related to short term satiety. Adding the macronutrient component also provides a background

response to test the effect of DF. I analyzed the interactions between DF and macronutrients to further investigate the potential additive effects of fermentation and macronutrients on hormonal production and food intake. Lastly, two rat models (Fischer 344 and Sprague Dawley) were used to examine whether the responses investigated in this thesis conserved within species.

Given the apparent association between higher DF intake and reduced weight gain, and the links between DF and PYY and GLP-1, macronutrient and PYY and GLP-1, and PYY and GLP-1 and food intake, I hypothesized that:

1) DF feeding increases basal levels of PYY and GLP-1.

2) Increased PYY and GLP-1 levels will enhance short term satiety.

3) Apart from the effect of DF, macronutrients will have an additive effect on short term satiety.

4) Chronic DF treatment will alter food intake, body weight, and body composition over the long term in rats.

5) Effects of WB and FOS diets will be similar because they supply equal amounts of fermentable material.

Chapter 2 of this thesis described the experimental protocols used to test the hypotheses. Chapter 3 and 4 described the results obtained from Fischer 344 and Sprague Dawley rats, respectively. The results from both rat models were discussed together in Chapter 5.

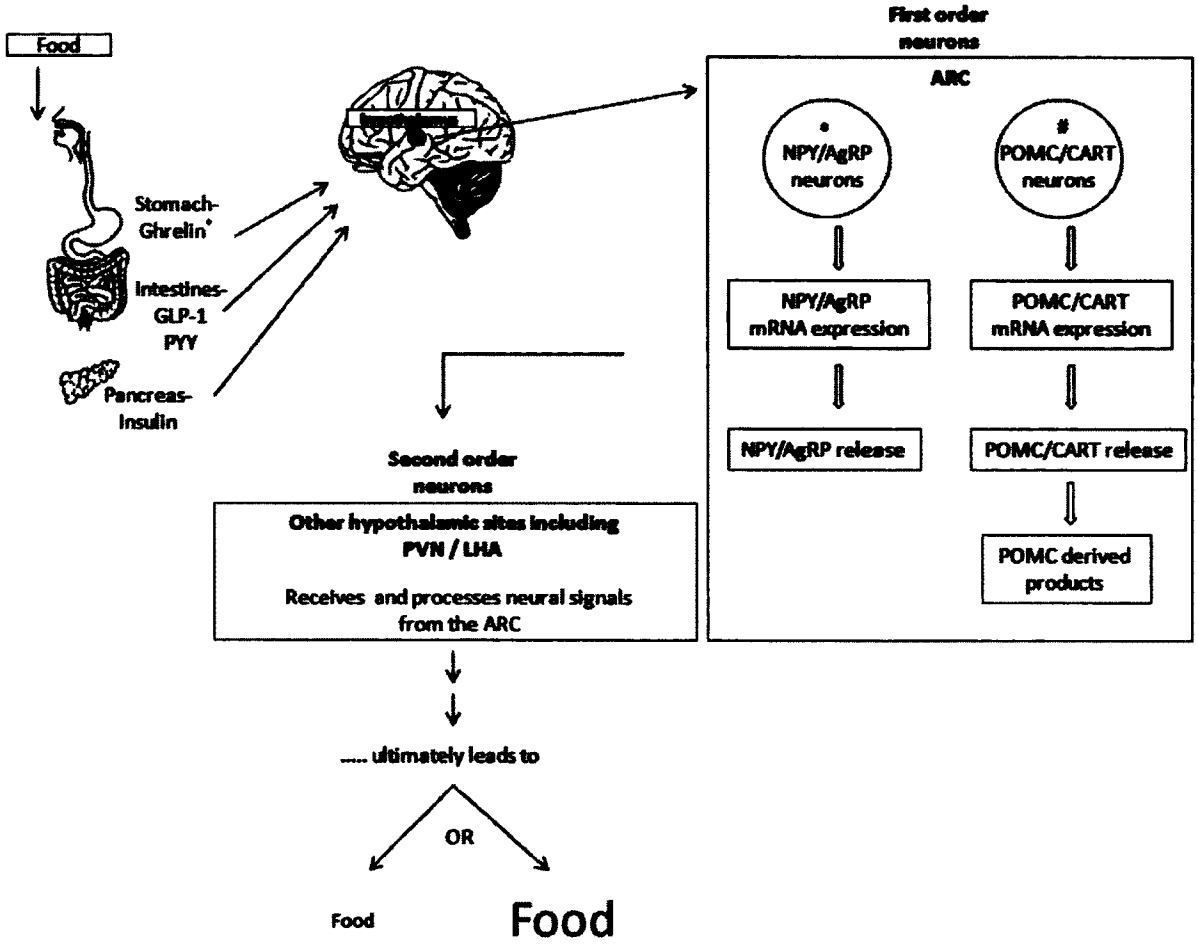


Figure 1.1: Schematic representation of the release and action of various hormones on the ARC neurons affecting food intake.

Chapter 2

Methods and Materials

The following procedures were followed and repeated for both cohorts: male Fischer 344 (F344) and Sprague Dawley (SD) rats.

Rats and Diets

All animal procedures were approved by the Health Canada Animal care committee and maintenance of rats followed the guidelines of the Canadian Council of Animal Care.

Male 7 weeks old rats (n=36, body weight: ~180g for Fischer and ~200g for Sprague Dawley) were purchased (Charles River, Kingston, NY) and housed individually in stainless steel wire bottomed cages in a climate controlled room (temperature, 22°C) with a 14:10 h light-dark cycle.

Acclimation to environment

During the environmental acclimation period (5 days), rats were fed a commercial rodent diet upon arrival for 2 days. Rats were divided into 3 energy-matched diet groups: control (modified AIN-93G diet), Fructooligosaccharides (FOS) (modified AIN-93G diet containing 5% FOS) or wheat bran (WB) (modified AIN-93G diet containing 10% WB) based on weight. Average body weights of each diet group were ± 5 grams. Rats were fed the assigned diet for 3 days with the exception that 5% FOS was mixed in with the control diet to minimize discomfort caused by high content of rapidly fermented fibre. During acclimation, rats were fed *ad libitum* 24 hours a day with free access to water. Diets were in pellet form, custom-made as Table 2.1 and purchased from Dyets Inc. Orafti P95 FOS was purchased from Quadra Chemicals and certified hard red wheat bran was purchased from AACC International.

Table 2.1. Composition of the diets

Diet ingredients	Control	10% WB	5% FOS
Casein	200.0	162.5	200.0
Corn Starch	376.0	355.7	367.9
Dextrinized Corn Starch	110.5	90.2	102.4
Sucrose	78.5	58.2	70.4
Soybean oil	70.0	62.3	70.0
AIN-93 mineral mix	35.0	35.0	35.0
AIN-93 vitamin mix	10.0	10.0	10.0
Choline Bitartrate	2.5	2.5	2.5
DL Methionine	3.0	3.0	3.0
Alphacel (wood cellulose)	114.6	0.0	86.9
Tert-butylhydroquinone	0.014	0.014	0.014
DF source:	None	WB	FOS
g to add:	0.0	220.8	52.0
Total Weight (g):	1000	1000	1000
Energy density (kcal/g):	3.70	3.70	3.70
Total protein	20	20	20
Total carbohydrate (less DF)	56.5	54.5	54.1
Total fat	7	7	7

Feeding phase: acclimation to assigned diets

After the initial environmental acclimation, rats were fed their respective diets for 3 weeks to stabilize the bacterial community. During this period, rats had free access to water and were fed *ad libitum* during dark cycle. Control diets were introduced to all rats from 0-2h upon the start of dark cycle then changed to treatment diets from 2-10h. This was to prepare them for the experimental period, where short term (0-2h) food intake was measured using only the control diet. This was to eliminate the effect of treatment diet on short-term food intake due to confounders such as taste/palatability. Food intakes were measured to the nearest 0.1g daily.

Adaptation to gavage

During the adaptation phase, rats were gavaged with water twice to acclimatize them to this procedure. Following gavage, food intake was measured at 0.5, 1, 2h and end of dark cycle. Food intake was also measured the next day without gavaging. This was repeated twice to ensure there was no difference in food intake caused by gavage.

Physical measurements

Body weight: body weights were recorded weekly until onset of nutrient preload experiments.

Body composition: percentage body fat was determined prior to the nutrient preload experiments and at the end of the study using an EchoMRI instrument.

Nutrient Preload Experiments

Rats were randomly given 4 preload solutions (water, glucose, whey protein, or canola oil) by gavage 30 min before the onset of the dark cycle. The experiment was designed so that every rat acted as its own control (the water preload). Preloads were 2.5 mL of water or 2.5 mL of total solution containing 0.75g of glucose or protein. Emulsion of canola oil with water was not possible, so 0.8 mL of canola oil was given. Only 12 rats were gavaged per day. Each preload was given on 2 occasions separated by at least a 1d washout. As illustrated in Figure 2.1, on the first occasion, subsequent food intake was measured at 30 min, 60 min, 120 min and at the end of the dark cycle. On the second occasion, 300 μL of tail vein blood was collected, 30 min after gavage, in chilled Microvette 200 serum tubes with clot activator (Sarstedt) containing: Dipeptidyl peptidase-4 inhibitor (DPPIV) (Millipore) and serine protease inhibitor, Pefabloc SC (Roche Applied Science) for the measurement of hormones. 1μL of each inhibitor was needed per 100 μL of blood collected. Tubes were inverted several times and kept on ice. Blood was allowed to clot for at least 30 min before centrifugation at 1000xg for 10 min. Serum aliquots were stored at -80°C until further analysis. The experiment ended when all rats had received all four types of preload.

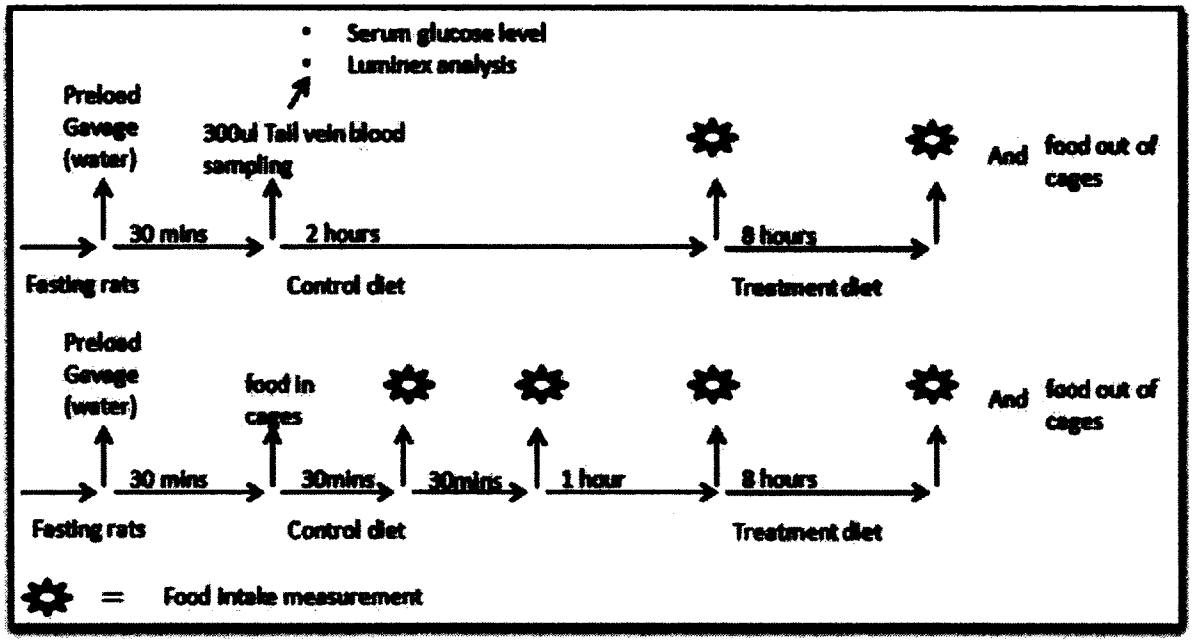


Figure 2.1. An illustration of a typical nutrient preload experiment.

Necropsy

At the end of the experiment, food-deprived rats were sacrificed by decapitation without sedation or anesthesia 30 min after gavage feeding either water or glucose (same amount as nutrient preload experiment). Trunk blood was collected in BD vacutainer SST tubes with clot activator for serum separation. Dipeptidyl peptidase-4 and Pefabloc SC were added immediately and blood samples were kept on ice for 30 min. and then centrifuged at 1000 x g for 10 min. Brain, white adipose tissue (epididymal), mesenteric lymph nodes and gastrointestinal tract (from stomach to rectum) were collected, weighted (cecum) and flash frozen in liquid nitrogen. The colon was rinsed with 1X phosphate saline buffer and cut in half for collection of proximal and distal colon. Because the

hormones of interest are only found in the mucosa and not in the muscular layers, the epithelial cells from the stomach, ileum, proximal and distal colon were collected from tissues held on an ice-cooled glass plate by scraping using a microscope slide. Tools used and glass plates were cleaned with 100% ethanol in between animals. Tissues and serum aliquots were kept in -80°C until further analyses.

Glucose measurements

Glucose in serum samples collected during nutrient preload experiments and necropsies was measured enzymatically using the GM9D analyzer (Analox instrument). The glucose oxidizing reagent (containing a hemolyzing agent) was air equilibrated and pumped to the reaction chamber. Oxidized glucose was converted to gluconic acid, and the rate of oxygen consumption (measured by oxygen electrode) was directly related to the amount of glucose in the sample. The rate of change was directly proportional to the glucose present in the serum. The electrode membrane was changed for each assay run to ensure that the maximum response was detected.

The glucose reagent was brought to room temperature and cycled through the reaction chamber four times. The analyzer was calibrated with a glucose standard of 8mmol/L until triplicate readings less than 0.2 mmol/L apart were obtained. A 5 μL aliquot of serum was injected and data was recorded. First ten samples were run in duplicates to ensure the readings were within 0.2 mmol/L. After that, duplicates were run every 10 to 15 reactions. Once finished, water was cycled through the reaction chamber to flush out the glucose reagent.

Hormone measurements

ELISA

Serum corticosterone levels were measured using a commercial ELISA kit (Cat#55-CORMS-E01, ALPCO). Manufacture's protocols were followed. Briefly, 10 μL of the standards and serum samples were loaded into the corticosterone antiserum coated wells followed by 100 μL of incubation buffer and 50 μL of corticosterone conjugated horseradish peroxidase. Samples were incubated for 2h on a plate shaker at 700 rpm at room temperature. The content of the wells were discarded by inverting the plate and washed four times with 300 μL of the diluted wash buffer (provided). A 200 μL aliquot of substrate solution was then added followed by 30 min. incubation in the dark. Lastly, 50 μL stop solution was added and the absorbance was determined immediately at 450 nm. The concentration of the corticosterone in the serum sample was inversely proportional to the absorbance.

Luminex

Insulin, active ghrelin, total PYY and active GLP-1 in serum samples collected during nutrient preload experiments were measured by xMAP Luminex technology using the Rat Gut Hormone Panel Milliplex MAP kits (Cat# RGT-88K, Millipore) on a LiquiChip 200 workstation (Qiagen). The Luminex technology used colour-coded beads coated with a specific capture antibody and two fluorescent dyes. Multiple targets, each with a specific fluorescent dye, were analyzed together using the detection antibody and a streptavidin-phycoerythrin conjugate (fluorescent dye on reporter molecule). The first

laser excited the dye identifying the target, followed by a second laser that excited phycoerythrin to quantify the fluorescent reporter signal.

The manufacture's protocols were followed. Briefly, Hormones of interest have individual vials containing each of the antibody and they were sonicated for 30 seconds and vortexed for 1 min. Aliquots of 150 µL of each vial was added together and brought to a final volume of 3 mL with bead diluents provided in the kit. Serial dilutions of standards were made from a stock standard. A 200 µL aliquot of assay buffer was added into each well on the microtiter filter plate (provided) and mixed on a plate shaker for 10 min at room temperature. The assays were dried by vacuum and 25 µL of standard, quality controls and samples were added to the appropriate wells. A 25 µL aliquot of assay buffer was added to the sample wells and 25 µL of serum matrix (provided) was added to the standard and quality control wells. A 25 µL aliquot of the mixed beads, vortexed intermittently, were added to each well. The plate was sealed, covered with a lid, and incubated with agitation on plate shaker for 18 hours at 4°C. Fluid was removed and washed 3 times with 200 µL wash buffer (stock buffer was provided). A 50 µL aliquot of detection antibody was added to each well; sealed, covered with lid, and incubate with agitation on plate shaker for 30 min at room temperature. Then 50 µL of streptavidin-phycoerythrin conjugate was added to each well; sealed, covered with lid, and incubate with agitation on plate shaker for 30 min at room temperature. Fluid was removed and washed 3 times with 200µl wash buffer. Finally, 100 µL of sheath fluid was added to each well and mixed for 5 min on plate shaker. The plate was read immediately.

Arcuate nucleus (ARC) extraction

To avoid thawing the brain that could result in RNA degradation, tools were chilled on dry ice and the procedures were carried out on dry ice. The hypothalamus was cut into 1mm coroneal sections using razors and a rat brain matrix (Zivic Instruments). Based on the anatomy shown in The Rat Brain Atlas 6th Ed (George Paxinos and Charles Watson), a bunted 16 gauge needle was used to micro-punch the areas where ARC was present. A plunger was used to eject the tissue into a 1.5ml tube. Samples were stored in -80°C until further analyses.

Total RNA extractions

All tubes and tips used were autoclaved prior to use. Total RNA extractions were isolated from frozen tissues (ARC, stomach, ileum, cecum, proximal and distal colon) using TRIzol reagent (Invitrogen Life Technology) followed by purification and DNAse treatment. All of the twelve samples from each tissue were extracted. Approximately 100 mg of the tissue was removed and collected in a 2 mL tube and homogenized with 1ml of TRIzol reagent using a Polytron PT 3000. Homogenized samples were incubated at room temperature for 5 min followed by addition of 0.2ml of chloroform and subsequent incubation at room temperature for 3 min. Samples were centrifuged at 12500 for 15 min at 4°C. The aqueous phase was removed to a new 1.5 mL tube and RNA was precipitated with 0.5 mL of 100% isopropanol overnight at -80°C. Samples were thawed on ice and centrifuged at 12500 rpm for 15 min at 4°C. The supernatant was removed and the RNA pellet was washed with 1 mL of 75% ethanol. Samples were centrifuged at 9800 rpm for

5 min at 4°C. After the ethanol supernatant was discarded, the RNA pellet was air dried at room temperature for 10 min or until the edge of the pellet started to become transparent.

RNA cleanup was done on all RNA extractions using the Qiagen RNeasy mini kit (Cat #74106) and RNase free DNase kit (Cat#79254). RNA pellets were resuspended in 100 µL of RNase-free water and 350 µL of Buffer RLT. The pellets were vortexed until the solution was clear followed by the addition of 250 µL of 100% ethanol. The mixture was then transferred to an RNeasy mini spin column (provided) and centrifuged at 10,000 rpm for 15 seconds. The flow-through was discarded. A 350 µL aliquot of Buffer RW1 was then added to the column and it was re-centrifuged at 10,000rpm for 15 seconds. Flow-through was discarded, 10 µL of DNase I and 70 µL of Buffer RDD were mixed first then added to the column, and followed by incubation at room temperature for 15 min. Buffer RW1 (350 µL) was added to the column and centrifuged at 10,000rpm for 15 seconds. Flow-through was discarded. Buffer RPE (500 µL) was added and centrifuged at 10,000 rpm twice; first time for 15 seconds and second time for 2 min. The column was spun again for 1 min without buffers. Finally, 50µl of RNase-free water was then added, incubated at room temperature for 5 min, and centrifuged at 10,000rpm for 1 min to elute the RNA.

The purified RNA was quantified by measuring the absorbance at 260/280nm using a NanoDrop 1000 spectrophotometer (Thermo Scientific). The quality of RNA was assessed by the analysis of A260/A280 ratio and gel electrophoresis (to determine the integrity of the 28S and 18S ribosomal RNA).

Exception

ARC RNA was extracted with the same procedure as above, but with half of the amount used for TRIzol reagent, chloroform, isopropanol and ethanol. In addition, 6.5 μL of 5 mg/mL linear acrylamide (Ambion Inc) was added to increase visualization of RNA. The RNA pellet was re-suspended in 15 μL RNase-free water. Due to the small volume of total RNA available, RNA cleanup was omitted and RNA quality was analyzed using Experion RNA StdSens Chips (Cat #700-7153, Bio-Rad) instead of agrose gel. The manufacture's protocols were followed. Briefly, A 600 μL aliquot of RNA gel (provided) was loaded into the spin filter tube and centrifuged at 1,500 x g for 10 min. Then 65 μL of filtered gel was mixed with 1 μL of RNA stain in a separately tube. 1 μL of RNA ladder and 3μl of RNA samples were denatured at 70°C for 2 min and then immediately cooled by ice for 5 min. A portion of the stained gel (9 μL) was loaded into the assigned well on the chip and was primed by the Experion priming station (Bio-Rad). Then 9 μL of each of the filtered gel and stained gel were loaded into the assigned well. Finally, 5 μL of loading buffer was loaded into sample and ladder wells followed by 1 μL of denatured ladder or samples. The chip was vortexed in the Experion vortex station (Bio-Rad) for 60 seconds. The chip was analyzed immediately on the Experison automated electrophoresis station (Bio-Rad). The RNA bands were visualized electronically.

Complementary DNA (cDNA) synthesis

First strand cDNA was synthesized from 1 μg of total RNA, diluted with Milli Q water to a final volume of 10 μL, using the High Capacity cDNA Reverse Transcription kit with RNase inhibitor (Applied Biosystem).The master mix per reaction was 2 μL of 10X RT buffer, 0.8 μL 100 mM dNTP, 1 μL reverse transcriptase, 1 μL RNase inhibitor, 3.2 μL water and 2 μL 10X RT random primers. The diluted RNA was added to the master mix for a final volume of 20 μL. cDNA synthesis was performed on a Peltier Thermo Cycler (Bio-Rad): 10 min at 25°C, 120 min at 37°C, followed by 5 min at 85°C and then kept at 4°C. Dilutions of cDNA (10^{-2}) were prepared with water and stored at -20°C in aliquots.

Gene Expression Analysis:

Real-time PCR was performed on a CFX96 Real-Time System (Bio-Rad) using Brilliant III ultra-fast SYBR green QPCR master mix (Agilent Technologies). Analyses included the expression of genes of interest and housekeeping genes, which were hypoxanthine phosphoribosyltransferase (HPRT) and ribosomal protein 10A (RPL10A). The genes of interest were: ghrelin in stomach; PYY and proglucagon (GLP-1 precursor) in ileum, cecum, proximal and distal colon; and NPY, POMC, CART, and AgRP in ARC. The sequences of the primers summarized in Table 2.2 were previously designed by Dr. A Aziz lab and purchased from Integrated DNA Technologies. The 10 μL q-PCR reaction consisted of 0.5 μL of Milli Q water, 5 μL of master mix, 0.5 μL of the gene specific primer mixture (0.2 μM final concentration of forward and reverse primer) and 4

μL of cDNA. The PCR conditions were: initial denaturation at 95°C for 3 min, followed by 35 cycles of denaturing at 95°C for 5 seconds and annealing at 58°C for 10 seconds. The final step was 95°C for 10 seconds and a melt curve was generated from 65°C to 95°C with 0.5°C increment for 5 seconds. Each gene of interest was run on the same plate in duplicate with a 'no RT' (RNA sample without reverse transcriptase) and a 'no template reaction' as negative controls. Plate-to-plate variation in response was controlled by running a pooled cDNA sample containing the gene of interest and housekeeping genes (for example, for stomach: HPRT, RPL10A and ghrelin were the targets of interest. Therefore, each plate had 3 additional reactions HPRT, RPL10A and ghrelin with the same cDNA). Gene expression analyses were done on Bio-Rad CFX Manager version 2.0 software.

Statistical Analysis

All data were reported as mean ± standard errors of mean (SEM). Sample sizes were n=11-12 for Luminex analysis and real time-PCR, and n=6 for ELISA. Effects of preload on energy consumption, levels of serum glucose and hormones were analyzed by Repeated Measures ANOVA. Body weight and body composition were analyzed by one-way ANOVA. Relative gene expression was analyzed by two-way ANOVA. Post-hoc tests (Tukey HSD) were performed only on significantly different data ($P<0.05$). Data were transformed by Box-Cox prior to ANOVA if the standard deviation appeared to be related to the sample mean.

Table 2.2 Primer sequences used in real-time PCR

Gene	Primer sequence
HPRT	Forward: 5'-TTGCTCGAGATGTCATGAAGGA-3' Reverse: 5'-AGCAGGTCAGCAAAGAACTTATAG-3'
RPL10A	Forward: 5'-AGCCATGAGCAGCAAAGTTTCACG-3' Reverse: 5'-AGAAGCGTTTGTCCTTCTGAGGGT-3'
Proglucagon	Forward: 5'-AGACCGTTTACATCGTGGCTGGAT-3' Reverse: 5'-TGTCTGGGAAGCTGGGAATGATCT-3'
PYY	Forward: 5'-AAACTGCTCTTCACAGACGACAGC-3' Reverse: 5'-TCTTCACCACTGGTCACAACCTT-3'
Ghrelin	Forward: 5'-AGCCCAGCAGAGAAAGGAATCCAA-3' Reverse: 5'-ATGCCAACATCGAAGGGAGCATTG-3'
NPY	Forward: 5'-CAAGGCTTGAAGACCCTTCCATGT-3' Reverse: 5'-AGGCAGACTGGTTTCACAGGATGA-3'
POMC	Forward: 5'-AACATCTTCGTCCTCAAAGAGCTG-3' Reverse: 5'-GTAGCAGAATCTCGGCATCTTCCA-3'
CART	Forward: 5'-ATGAGAAGAAGTACGGCCAAGTCC-3' Reverse: 5'-CCCTTCACAAGCACTTCAAGAGGA-3'
AgRP	Forward: 5'-TTGGCAGAGGTGCTAGATCCA-3' Reverse: 5'-AGGACTCGTGCAGCCTTACAC-3'

Chapter 3

Results

Fischer 344 rats

Rats were divided into three diet groups: control (CTR), 5% fructooligosaccharides (FOS) and 10% wheat bran (WB). The four nutrient preloads were 2.5 mL water, 0.75 g glucose or protein in 2.5 mL water, and 0.8 mL canola oil. The energy density of glucose, protein, and oil preloads were 3, 2.8, and 6.75 kcal, respectively.

Physical measurements and long term food intake

At the start of the study, F344 rats weighted around 180 g at 7 weeks old (Figure 3.1). Body weight gain was more rapid until week 4 and then slowed. Percentage body fat (Figure 3.2) was similar across diet groups throughout the study and an influence of diet on body weight or body composition was not observed. Rats gained less weight as they grew older, and weight gain slowly decreased towards the end of study (Figure 3.3 A). Weekly energy consumption was roughly the same until week 4 and 5 where energy intake dropped significantly in all three groups. It was then constant at the new level till the end of study (Figure 3.3B). Overall, the FOS group consumed significantly less energy than WB. Energy consumption in the CTR group was not different from FOS or WB. Food efficiency was determined by dividing weekly weight gain by energy consumption. Energy efficiency was higher when rats were growing rapidly (weeks 1-4) and decreased as weight gain slowed towards the end of the study (Figure 3.3 C). Because the FOS group consumed significantly less energy than WB, but gained roughly the same amount of weight, it appeared that rats utilized the FOS diet more efficiently than WB (Figure 3.3C).

The effect of macronutrient preloads on short term satiety

Energy compensation after macronutrient preloads feeding was studied during the first 2 hours post gavage when all rats were given control diet. Water gavaged rats served as the control. Total energy intakes over the 10 h dark cycle were also monitored. Preloads were given 30 minutes prior to exposing the rats to food. The variability in food intake patterns among rats was large, so that a repeated measures ANOVA analysis was used to account for the rat-to-rat variability.

Figure 3.4 shows energy consumption after 30 min, 60 min, 120 min, 8 h (remaining dark cycle), and 10 h (daily total). Preloads affected energy consumption in all cases at 30, 60, and 120 min (Figure 3.4A-C; $p<0.001$). At 120 min (Figure 3.4C), however, energy consumption after a glucose preload was significantly higher than that after protein or oil ($p<0.05$). No differences were observed between protein and oil. No differences among rats fed different diets at 30, 60 or 120 min was observed.

After 120 min, rats were switched back to their experimental diets for the rest of dark cycle (Figure 3.4 D). Energy consumption was similar after water, glucose and protein preloads, but oil differed significantly compared to water or protein ($p<0.05$). Rats fed the FOS diet had significantly less energy intake than CTR or WB ($p<0.05$). Overall, daily energy consumption post preload (Figure 3.4 E) was significantly lower in rats fed the oil preload compared to all others ($p<0.001$) and the energy consumption after the protein preload was significantly lower than after the water preload ($p<0.05$). Once again, rats in the FOS group consumed significantly less energy than those in the CTR or WB groups ($p<0.05$). The effect of diet and preload were observed for daily total energy consumption including preloads (Figure 3.4 F): FOS diet had significantly lower

than CTR or WB diet ($p<0.05$) and oil preload was also significantly lower than water preload ($p<0.05$). There was no significant diet X preload interaction observed at any time point.

Real-time PCR analyses on mRNA hormone levels

Rats were gavage fed with either 2.5 mL water or 0.75 g glucose in 2.5 mL water 30 minutes prior to sacrifice. All genes of interest were normalized to 2 housekeeping genes: HPRT and RPL10A.Water gavaged and CTR diet fed rats acted as control in the analysis and the values were normalized to 1.0.

Hypothalamus arcuate nucleus hormones

NPY and AgRP are orexigenic peptides whereas POMC and CART are anorexigenic peptides. Therefore, the fasted rats are expected to have higher NPY and AgRP mRNA expressions and lower POMC and CART mRNA expressions (see introduction for more details). However, Figure 3.5A showed that glucose gavage tended to increase AgRP ($p=0.08$). Relative AgRP mRNA levels after glucose preload were observed be at least 2 times higher than water in both FOS and WB groups, although this did not reach statistical significance. The FOS fed rats had twice the relative AgRP mRNA levels as compared to WB-fed rats ($p<0.05$). No effect was observed for POMC, CART or NPY (Figure 3.5 B-D).

Lower gastrointestinal tract

PYY and GLP-1 (derived from PGC) are gut hormones released postprandially and are produced in the lower GI tract. Therefore, PYY and GLP-1 secretions are stimulated by exposure to nutrient availability in the GI tract and so are expected to be affected by fermentation events there as well. The buildup of those gut hormones are associated with reduced food intake [45]. Thus, I expect the hormone levels to be higher when both nutrient and fermenatable dietary fibre is present. Since the total fermentation levels were similar for both diets, one would expect the same response.

Relative PGC mRNA levels were unaffected by preload or diet in ileum (Figure 3.6A). Relative levels of PYY mRNA in ileum (Figure 3.6B) were significantly higher in CTR diet fed rats compared to WB fed rats (by 1.4 times, $p<0.05$). In the cecum, the rats fed the FOS diet had PGC mRNA levels that were 2 times higher than rats fed CTR or WB (Figure 3.6C-D, $p<0.001$). Glucose preload increased relative PGC mRNA level slightly across the three diets but not by much (Figure 3.6C). The effect of the glucose preload on PYY mRNA levels in rats fed FOS diets was almost 2 times higher when compared to that in rats fed a water preload (Figure 3.6D) but no other effect was observed . The mRNA levels of both PGC and PYY had similar expression pattern in CTR and WB group. As a result, although there was no significant effect on diet and preload interaction, the interaction of glucose and FOS diet had an indisputable additive effect on relative PYY mRNA. No significance was observed in proximal colon for either PYY or PGC (Figure 3.6 E-F). Relative PGC mRNA level after glucose preload was about 1.6 times higher than water in distal colon (Figure 3.6 G, $p<0.05$); but such

effect was not observed for PYY (Figure 3.6 H). No diet effect was observed for either gene of interest in the distal colon.

Stomach

Ghrelin is primarily produced by the endocrine cells of mucosa in the stomach fundus. Ghrelin is often accepted as the hunger signal where it is increased during the fasting state and is reduced following food intake [20,21]. No effect of gavage or diet on ghrelin mRNA expression was observed in this study (Figure 3.7). Thus: diet and preload had no affect on ghrelin mRNA levels.

Cecum size

Fermentation and diet affected the weight of the cecum and the cecal contents (Figure 3.8). Both the cecum and cecal content were significantly larger in rats fed the FOS diet as compared to those fed CTR or WB diets ($p<0.001$).

Distribution profile of relative mRNA in lower gastrointestinal tract

All mRNA expressions were done with 1 μg of total RNA. Each mRNA was normalized to its own housekeeping genes. The software then pooled all the mRNA expressions from the same tissue together as a "biological set". Therefore the mRNA ratio of each tissue was normalized by taking into account the relative amount of housekeeping genes per ug RNA for each tissue section.

PGC (Figure 3.9A)

The relative PGC mRNA level was the highest in the ileum compared to cecum, proximal colon and distal colon. Cecum, proximal colon and distal colon had similar level of PGC expression overall, but FOS diet in the cecum being noticeably higher than the rest.

PYY (Figure 3.9B)

The relative PYY mRNA levels were similar in the ileum, proximal colon and distal colon. Cecum appeared to have the lowest amount of PYY mRNA expression per 1μg of total RNA. Rats fed the FOS diet expressed higher level of PYY in the cecum that was similar to the levels of expressions as the other tissues, rats fed the CTR and WB diet had a notably much lower expression of PYY in the cecum.

Luminex analyses of circulating hormones in serum

The level active ghrelin, insulin, active GLP-1 and total PYY in the serum were measured in tail vein samples obtained 30 minutes after the macronutrient preloads.

The relative levels of active ghrelin among preloads were: water, glucose >> protein, oil. Levels of active ghrelin were similar in rats fed water and glucose preloads but significantly lower in those fed protein and oil preloads (approximately 0.7 X control group; $p<0.001$; Figure 3.10A). There was no overall diet effect on active ghrelin levels. There is no statistical diet X preload effect.

The relative levels of insulin among preloads were: glucose > protein > oil > water. Both glucose and protein induced approximately 1.5 times the value in rats gavaged with water (Figure 3.10B, $p<0.05$). The level of insulin after the oil preload was higher, but not significantly, than water. No effect of diet or diet X preload interaction was observed.

No effect of preload or diet on active GLP-1 secretions was observed (Figure 3.10C).

Both diet and preload had significant effect on total PYY hormone level. The relative levels of total PYY among preloads were: oil >> water, protein, water (Figure 3.10D). The relative levels of total PYY among diets were: FOS >> CTR, WB. Rats fed the FOS diet had circulating total PYY levels that were 1.5 X those of rats fed the CTR or WB diets ($p<0.05$). The levels of total PYY after the oil preload were 2 X those of rats fed the other preloads ($p<0.001$). The levels of total PYY were similar after feeding oil preloads regardless of diets. Therefore, no additive effect of FOS and oil was observed as oil seemed to increase total PYY independent of diets. Oil preload alone seemed to be able to override the FOS effect on PYY.

Correlations between hormones and energy consumption

Relationships between levels of circulating hormones and energy consumption at different time points were studied as well as the relationship between circulating hormones after the same preload. The time points studied for energy consumption were first hour, second hour and first 2 hours. Only the relationships showing a significant correlation are presented.

After water preload, insulin was positively and linearly proportional to energy intake during the first hour (Figure 3.11A, $p<0.05$) and the first 2 hours (Figure 3.11B, $p<0.01$). Insulin was also strongly correlated with ghrelin after water and glucose preloads (Figure 3.12 A-B; $p<0.05$ and $p< 0.001$, respectively), as well as with active GLP-1 after water preload (Figure 3.12 C; $p<0.005$). Active GLP-1 was also strongly correlated with total PYY after water, glucose and protein preloads (Figure 3.13 A-C, $p<0.001$). No correlation between active GLP-1 and total PYY was observed after oil preload. These relationships seemed to be independent of diet.

Serum glucose and corticosterone

The effect of macronutrient preloads on serum glucose levels was studied using two different samples: tail vein blood samples taken 30 minutes after each preload, and trunk blood collected during necropsy. It was surprising that serum glucose was constant throughout the experiment in tail vein samples despite gavaging a 2.7g/kg body weight solution (Figure 3.14A), the procedure commonly employed in these types of experiments. However, serum glucose responded to diet and gavage when measured in necropsy samples (Figure 3.14B). Study has shown that stress induce blood glucose significantly in rats [125]. It is possible that stress induced during tail vein sampling may have influenced glucose levels. Rats being temporary restrained during such procedure could also present an additional stressor, which may be the cause of the discrepancies observed in serum glucose level between preload experiment and necropsy. Corticosterone is a hormone released in response to stress and is often used as the stress indicator in rodent. Different methods of blood sampling, such as tail vein nick or

sacrifice by decapitation, have been shown to alter the level of corticosterones [126]. Therefore, the level of corticosterone was measured in serum samples obtained from rats after gavage with water. Repeated measures ANOVA analysis showed that the corticosterone levels were significantly higher after sacrifice compared to after tail vein bleeding (Figure 3.15, $p<0.05$). The effect of diet was not significant, FOS group consistently had higher level of corticosterone and especially evident at necropsy where FOS group had 1.25 and 1.45 times higher corticosterone level compared to CTR and WB, respectively.

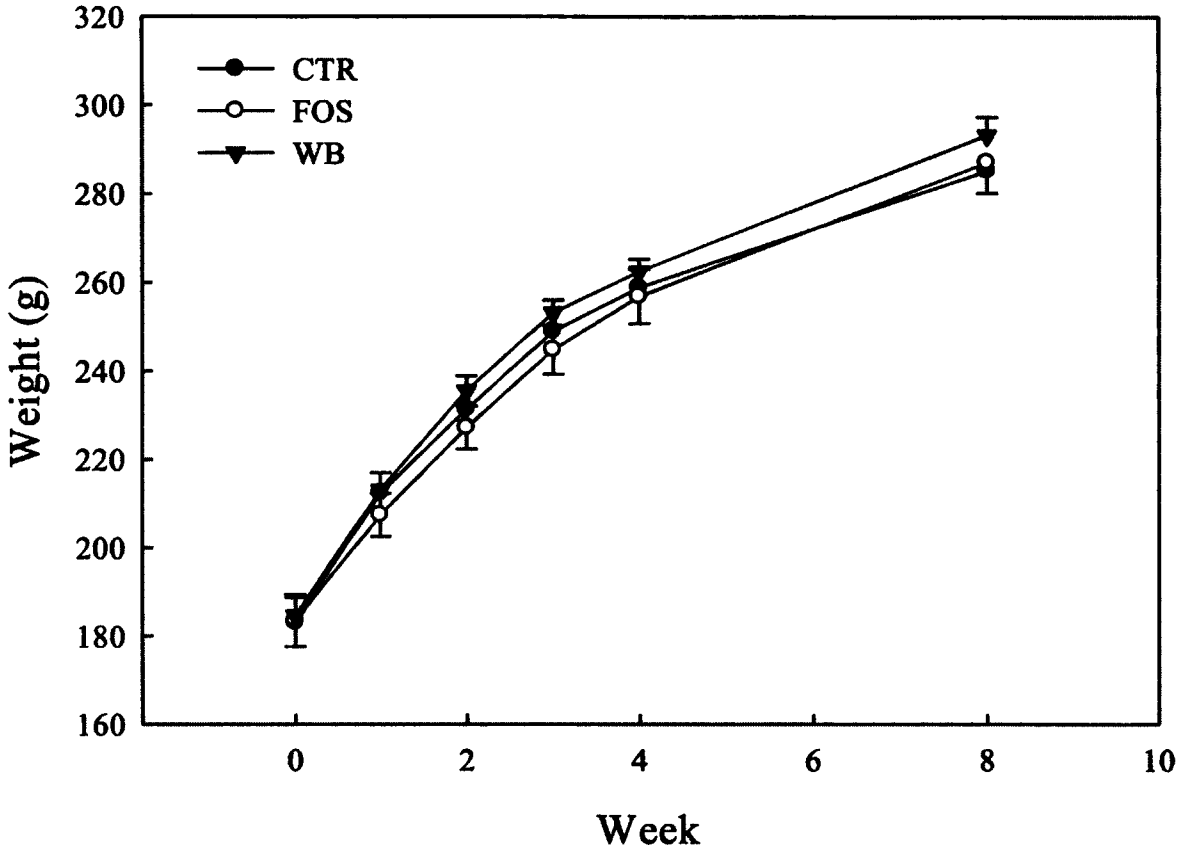


Figure 3.1: Body weight of F344 rats fed one of three experimental diets. The diets were control (black circles), FOS (white circles) and WB (inverted grey triangles). Rats were 7 weeks old at week 0. Body weights were not measured during the nutrient preload period (weeks 6-8). Values represent mean ± SEM (n=12 per diet group). Data were analyzed using one-way ANOVA. No significant differences were detected at the $p < 0.05$ level.

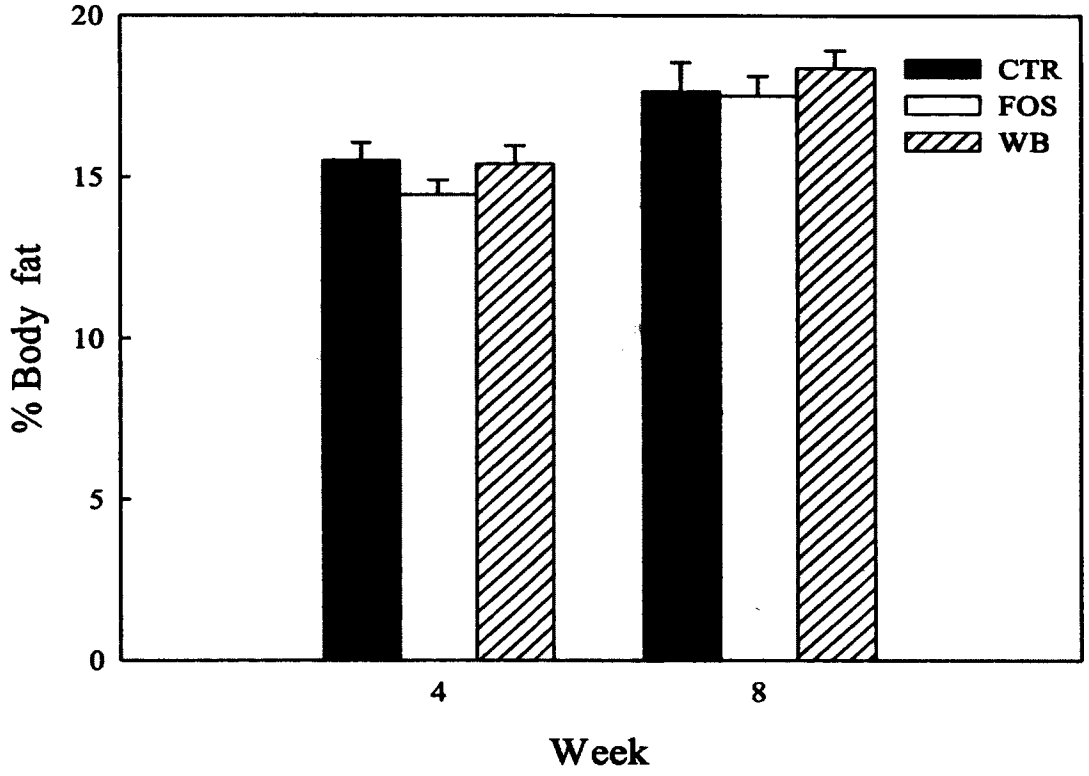


Figure 3.2: Body composition of F344 rats fed one of the three experimental diets. The diets were control (black bars), FOS (white bars) and WB (lined bars). Percentage body fat was determined based on the weight of fat mass analyzed by EchoMRI instrument over the body weight. Body composition was done immediately after the body weight measurements; two time point of body composition measurements were before the nutrient preload experiment and before necropsy. Values represent mean ± SEM (n=12 per diet group). Data were analyzed using one-way ANOVA. No significant differences were observed at the $p < 0.05$ level.

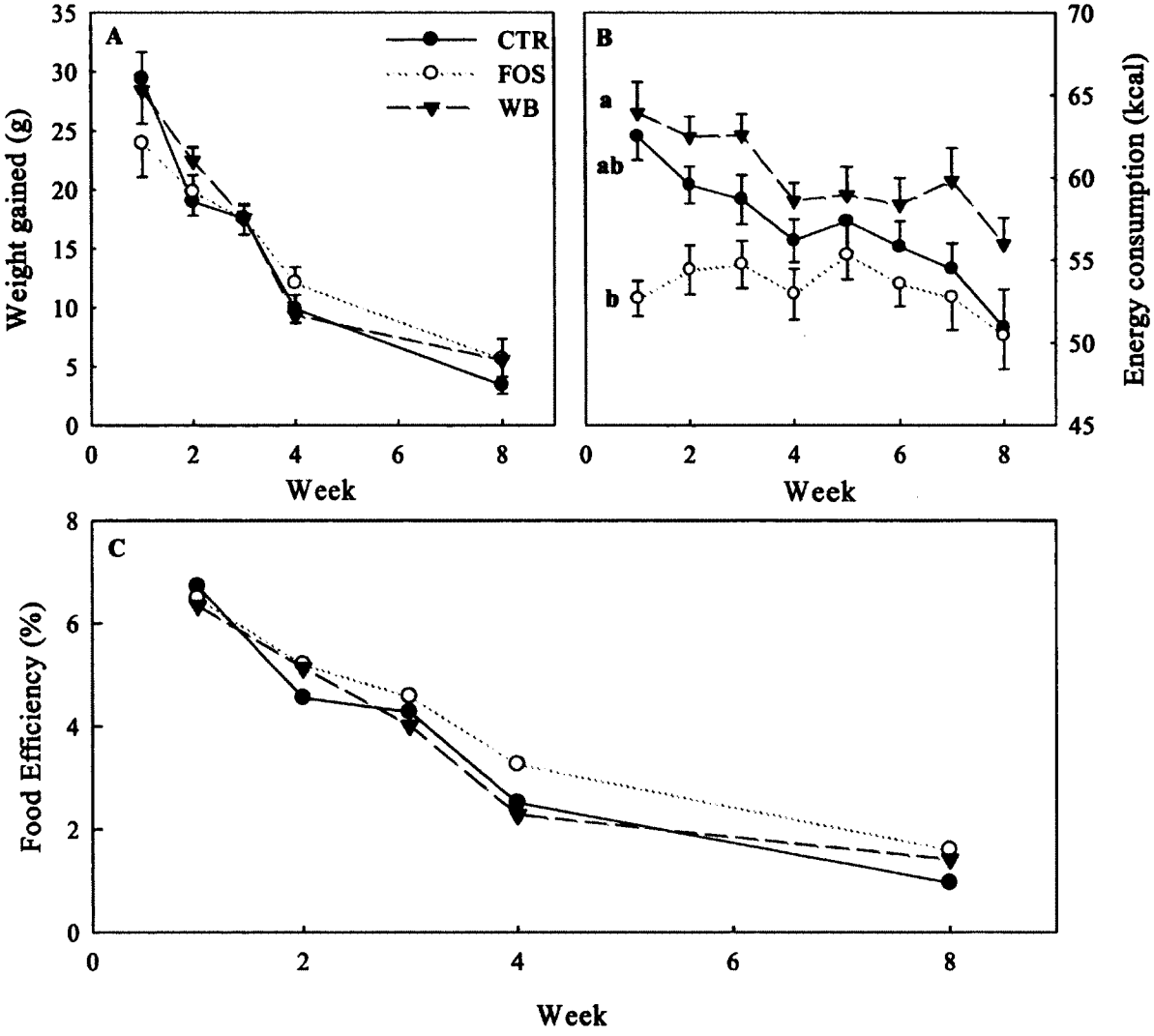
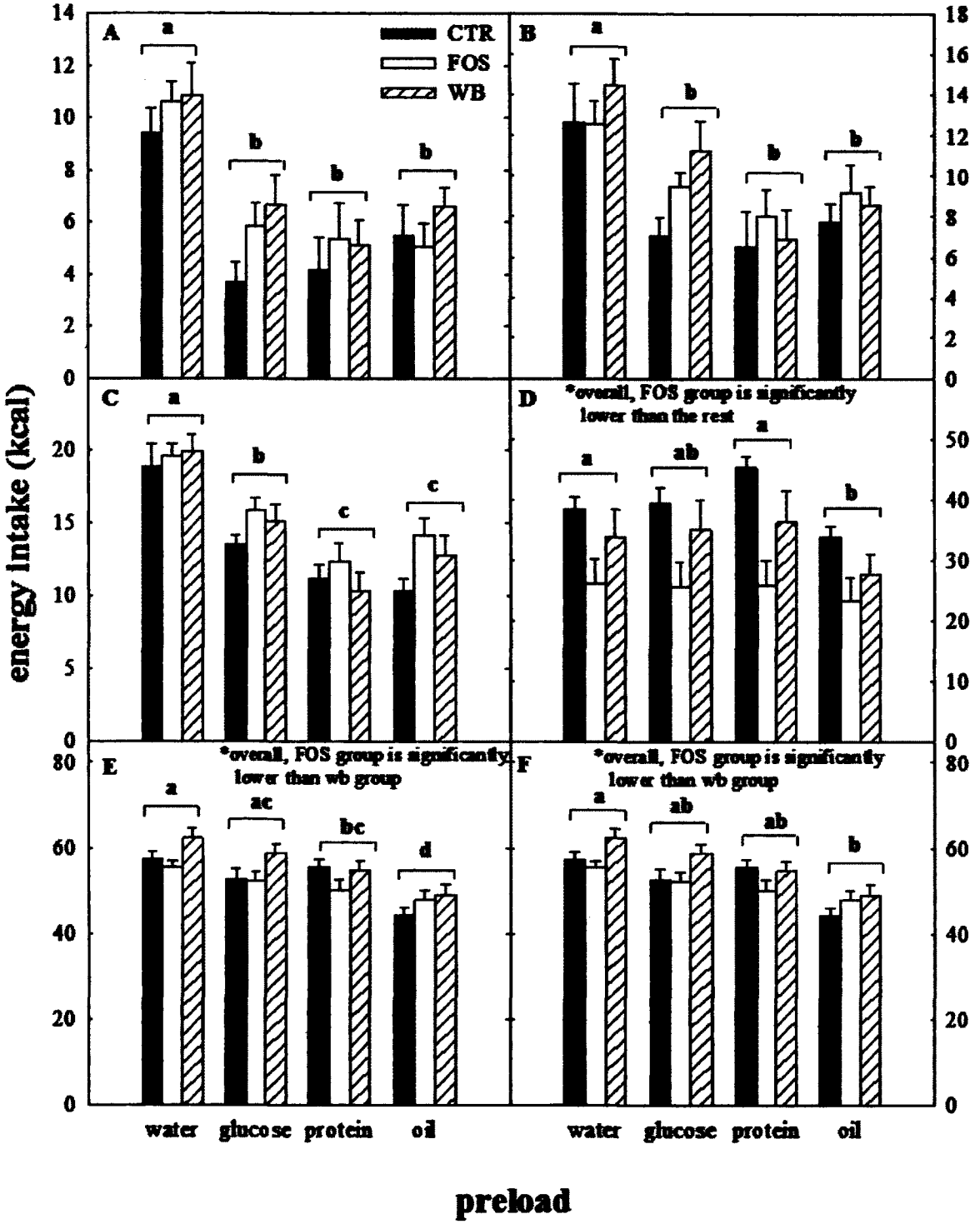


Figure 3.3: Food efficiency of F344 rats fed one of the three treatment diets. The experimental diets were control (black circles), FOS (white circles) and WB (inverted grey triangles). Food efficiency was calculated from measurements of body weight gain and energy consumption (g gained/kcal consumed * 100%). Panel A: average weekly weight gain; panel B: average daily energy consumption by week; and panel C: average weekly food efficiency. Values represent mean ± SEM (n=12 per diet group). Data were analyzed using repeated measures ANOVA; $p < 0.05$ was considered as significantly different. Diet groups sharing the same letter were not significantly different.

Figure 3.4: Energy consumption of F344 rats at various time points after gavage feeding with one of the four macronutrient preloads 30 minutes prior to the onset of dark cycle. The four nutrient preloads were 2.5 mL water, 0.75 g of glucose or protein in 2.5 mL water and 0.8 mL oil. Three treatment diets were control (black bars), FOS (white bars) and WB (diagonal lined bars). All rats were fed with control diet for the first 2 h to eliminate possible confounders that might affect food intake. Energy consumption was calculated by multiplying amount of food intake measured (in grams) by the caloric density of the diet (3.7 kcal/g). Panels A-E represent the energy intake of the consumed diet only. Panel F represents the daily total energy intake including the energy contribution of the preloads. Panel A: 0-0.5 h; panel B: 0-1 h; panel C: 0-2 h; panel D: 2-10 h (treatment diets); panel E: daily total without energy consumed from preload; and panel F: daily total including energy density obtained from preload. Values represent mean ± SEM (n=12 per diet group). Data were analyzed using repeated measures ANOVA with diet and preload as factors; $p < 0.05$ was considered as significantly different. Diet groups sharing the same letter were not significantly different. * denotes that FOS group consumed significantly less energy compared to control or WB groups.

Figure 3.4


A
B
C
D
E
F
CTR
FOS
WB
energy intake (kcal)
*overall, FOS group is significantly lower than the rest
*overall, FOS group is significantly lower than wb group
water
glucose
protein
oil
preload

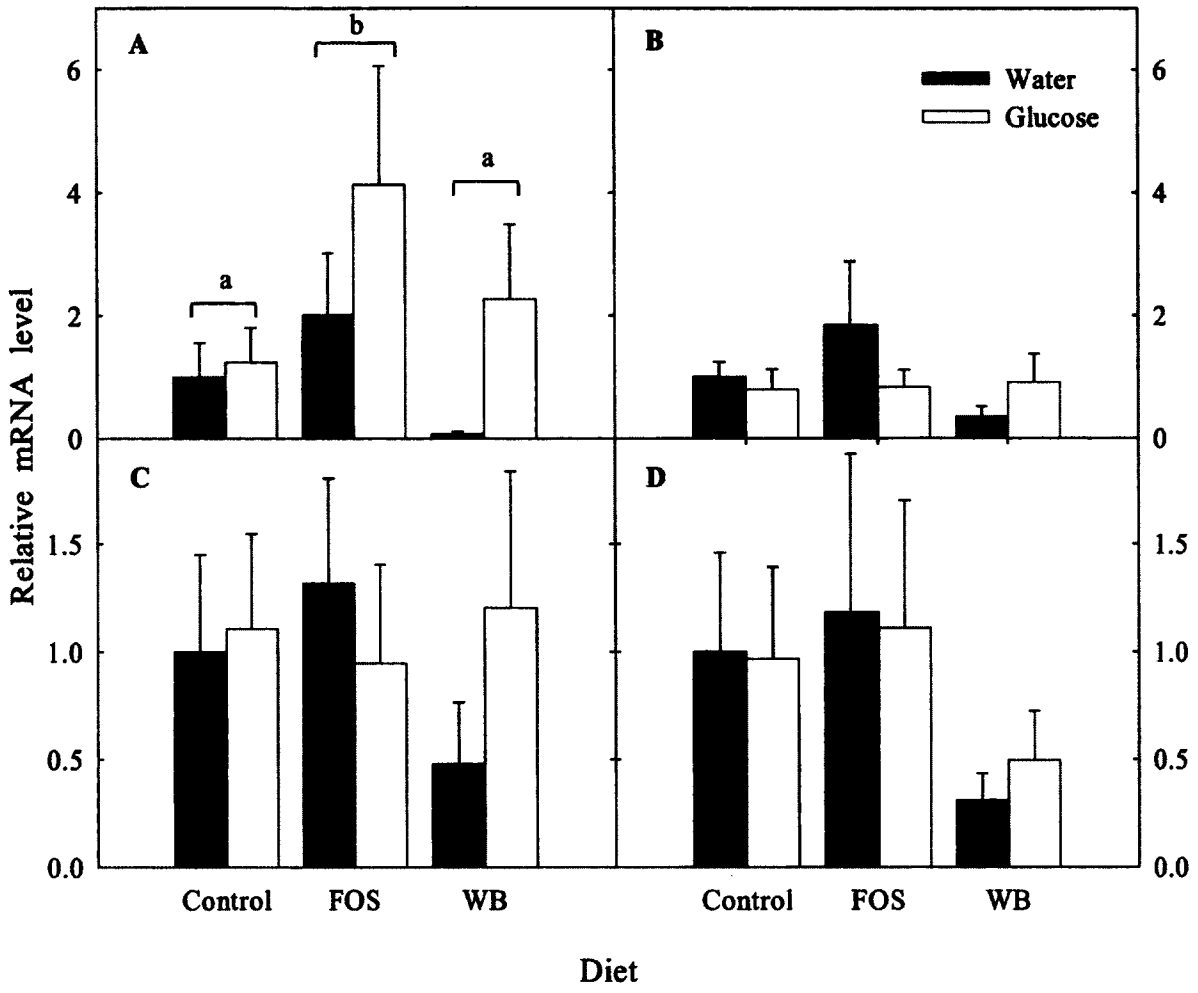
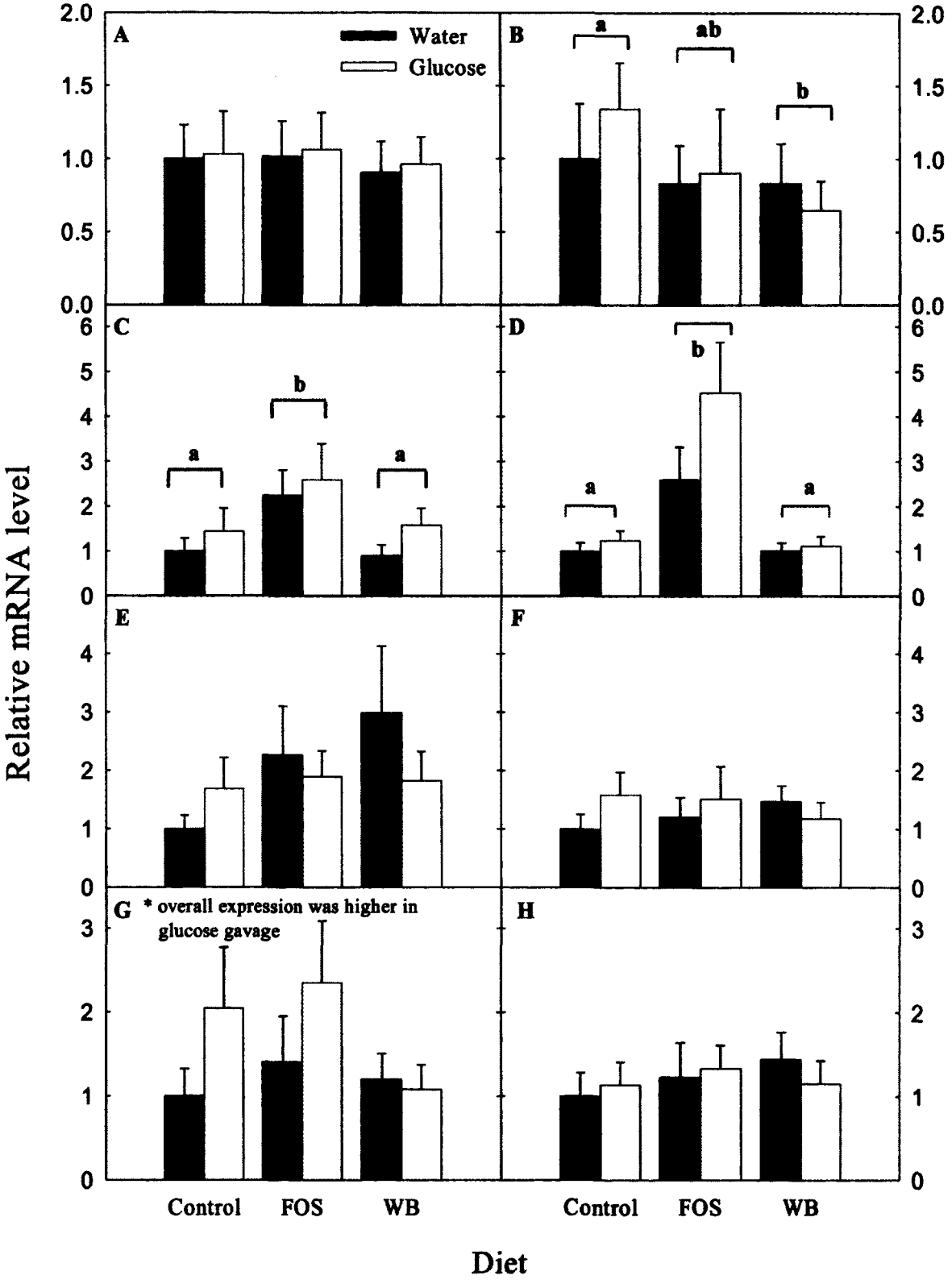


Figure 3.5: Real-time PCR analysis of mRNA hormone levels in hypothalamus arcuate nucleus (ARC) of F344 rats fed one of the three diets measured after necropsy. Rats were gavage fed preload containing either 2.5 mL water (black bars) or 0.75 g of glucose in 2.5 mL water (white bars) 30 minutes prior to sacrifice. Panel A: AgRP; panel B: POMC; panel C: CART; and panel D: NPY. All values are relative to water-gavage, control-fed rats (set to 1.0). Values represent relative mean ± SEM (n=6 per preload, n=12 per diet group). Data were analyzed using two-way ANOVA with type of diet and preload as categorical predictors; $p < 0.05$ was considered as significantly different.

Figure 3.6: Real-time PCR analysis of mRNA hormone levels in the lower gastrointestinal tract of F344 rats fed one of the three diets measured after necropsy. Rats were gavage fed preload contained either 2.5 mL water (black bars) or 0.75 g of glucose in 2.5 mL water (white bars) 30 minutes prior to sacrifice. Panel A: PGC in ileum; panel B: PYY in ileum; panel C: PGC in cecum; panel D: PYY in cecum; panel E: PGC in proximal colon; panel F: PYY in proximal colon; panel G: PGC in distal colon; and panel H: PYY in distal colon. All values are relative to water-gavage, control-fed rats (set to 1.0). Values represent relative mean ± SEM (n=6 per preload, n=12 per diet group). Data were analyzed using two-way ANOVA with diet and preload as categorical predictors; $p < 0.05$ was considered as significantly different. Only panels with significant differences observed were labeled with letters or asterisk. Diet groups sharing the same letter were not significantly different. * denotes glucose gavage was significantly different from water gavage.

Figure 3.6


A
Water
Glucose
B
a
ab
b
C
a
b
a
D
a
b
a
E
F
G
* overall expression was higher in glucose gavage
H
Relative mRNA level
Control
FOS
WB
Control
FOS
WB
Diet

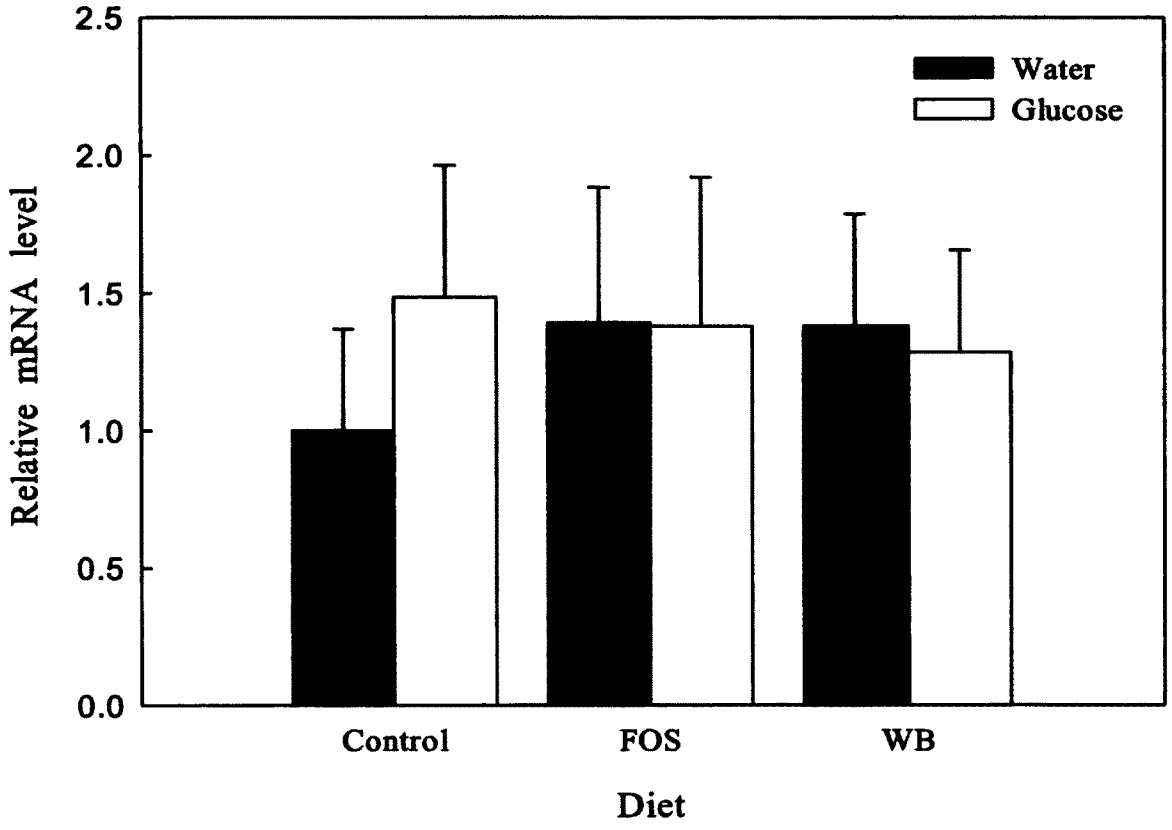


Figure 3.7: Real-time PCR analysis of mRNA levels of ghrelin in the stomach of F344 rats fed one of the three diets measured after necropsy. Rats were gavage fed preload containing either 2.5 mL water (black bars) or 0.75 g of glucose in 2.5 mL water (white bars) 30 minutes prior to sacrifice. All values are relative to water-gavage, control-fed rats (set to 1.0). Values represent relative mean ± SEM (n=6 per preload, n=12 per diet group). Data were analyzed using two-way ANOVA with diet and preload as categorical predictors; $p < 0.05$ was considered as significantly different.

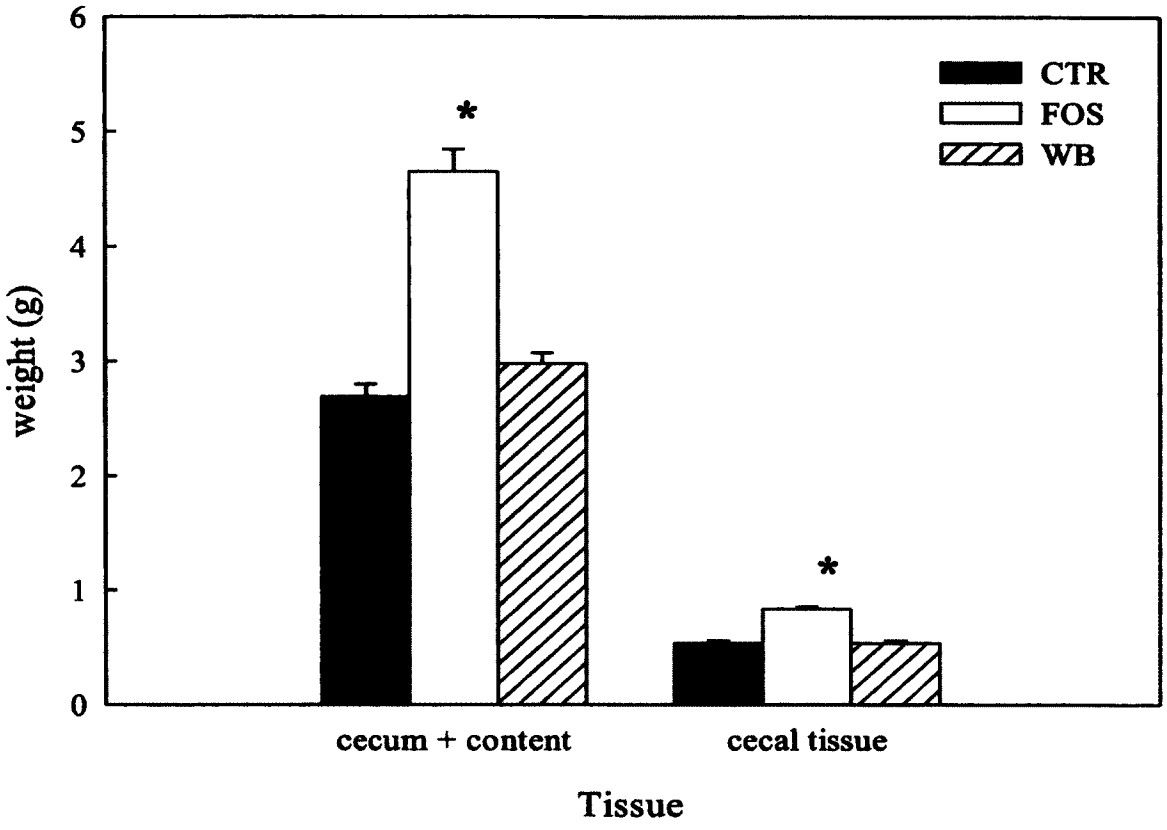


Figure 3.8: Weight of cecal tissue of F344 rats fed one of the three diets as measured post during necropsy. The experimental diets were control (black bars), FOS (white bars) and WB (diagonal lined bars). Values represent mean ± SEM (n=12 per diet). Data were analyzed using one-way ANOVA; $p < 0.05$ was considered as significantly different. Asterisks denote that FOS group had significantly different compared to the CTR or WB group with or without cecal contents.

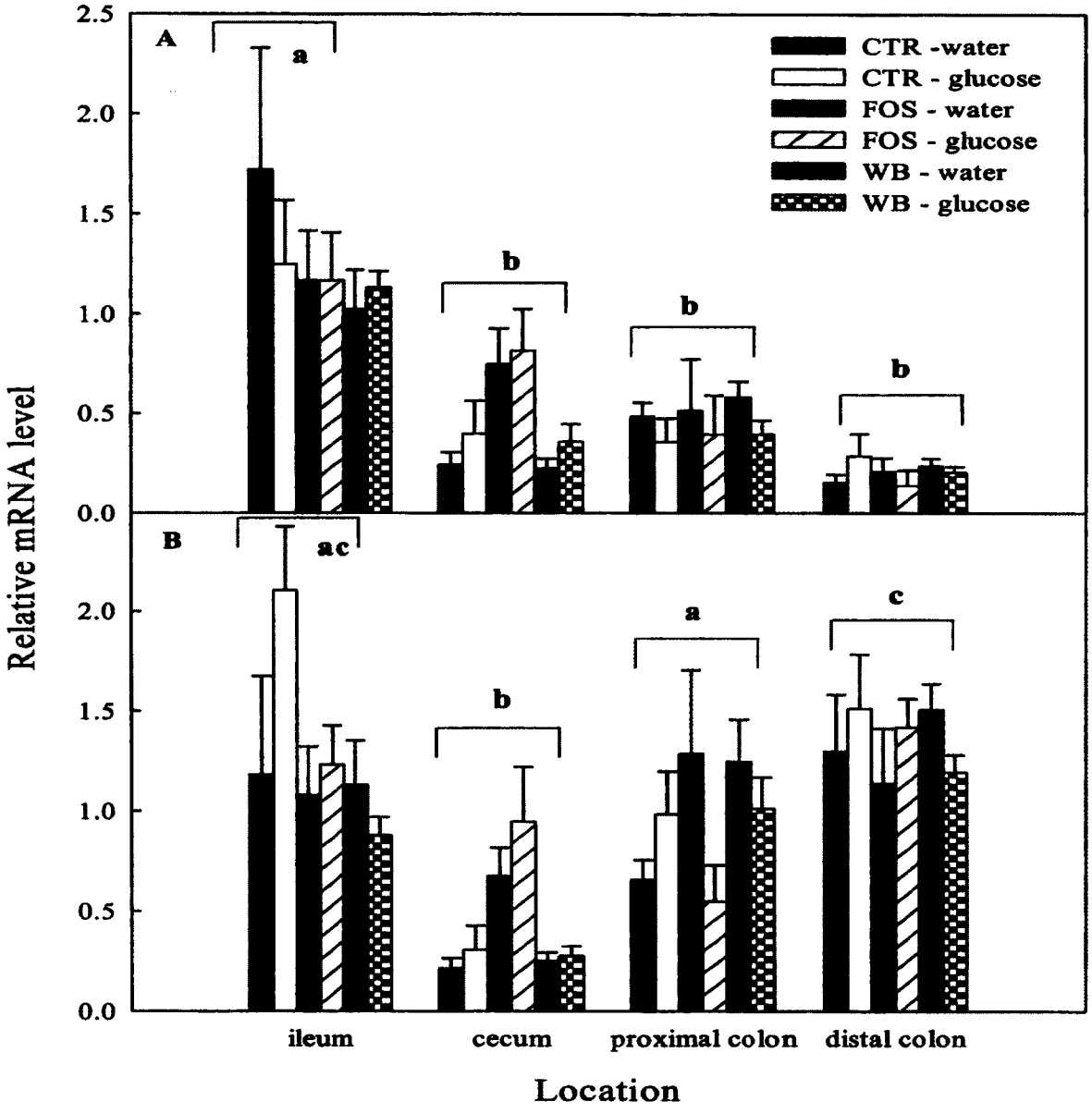


Figure 3.9: Distribution of mRNA hormone levels analyzed by real-time PCR in the lower gastrointestinal tract of F344 rats fed one of the three diets measured after necropsy. Rats were gavage fed preload containing either 2.5 mL water (grey bars) or 0.75 g of glucose in 2.5 mL water (white bars) 30 minutes prior to sacrifice. The treatment diets were: control (empty bars), FOS (diagonal lined bars) and WB (checked bars). Panel A: distribution of PGC and panel B: distribution of PYY. Representative histograms showing mean values of relative expression across tissues normalized to housekeeping genes ± SEM (n=6 per preload, n=12 per diet group). Tissues sharing the same letter were not significantly different.

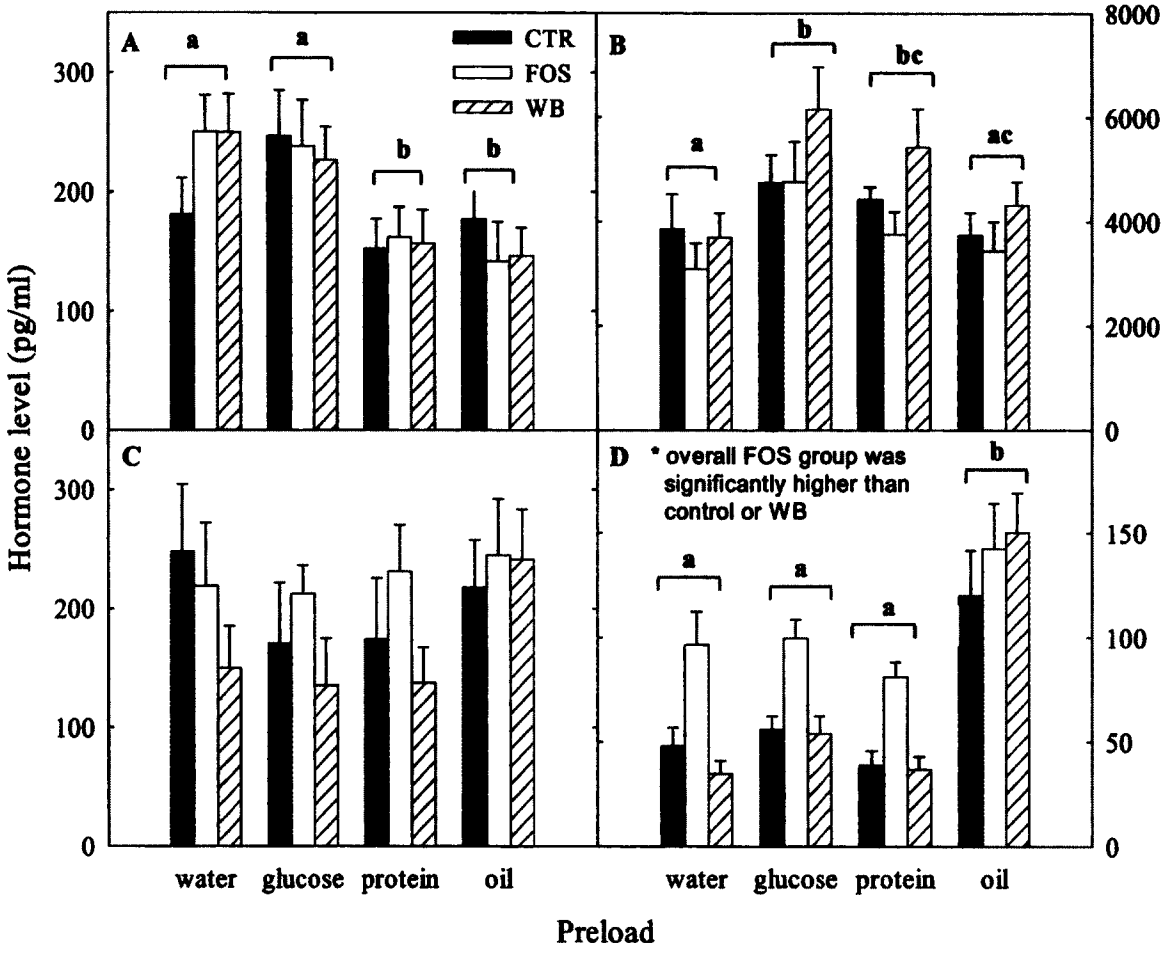


Figure 3.10: Luminex analysis of serum hormone levels of F344 rats fed one of the three diets measured 30 minutes after gavage. The experimental diets were control (black bars), FOS (white bars) and WB (diagonal lined bars). The four nutrient preloads were 2.5 mL water, 0.75 g glucose or protein in 2.5 mL water and 0.8 mL oil. Panel A: active ghrelin; panel B: insulin; panel C: active GLP-1and panel D: total PYY. Values represent mean ± SEM (n=12 per diet group). Data were analyzed using repeated measures ANOVA with diet and preload as factors; $p < 0.05$ was considered as significantly different. Only panels with significant differences observed were labeled with letters or asterisks. Diet groups sharing the same letter were not significantly different. * denotes FOS diet were significantly different from both control and WB diets.

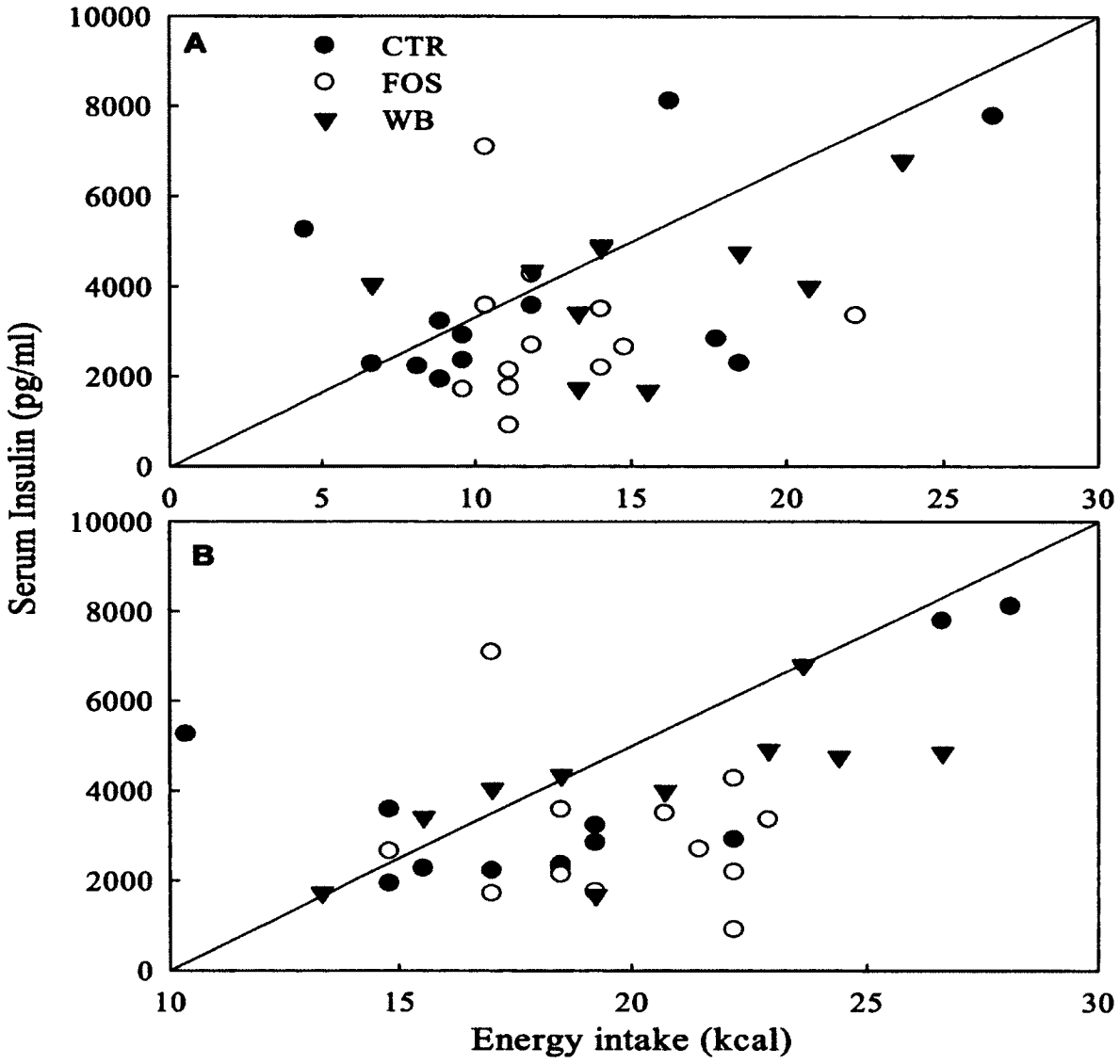


Figure 3.11: Relationship between circulating insulin levels and energy intake over the first 2 h in F344 rats fed one of the three diets after 2.5 mL of water preload. The experimental diets were control (black circles), FOS (white circles) and WB (inverted grey triangles). Levels of serum insulin were determined 0.5 h after gavage by Luminex on separate days. Panel A: first hour of feeding and panel B: first 2 hours of feeding. Food intake (g) was converted to energy intake by multiplying by the energy density of the diets (3.7kcal/g). Values represent mean (n=12 per diet group). Data were analyzed using simple regression. Diagonal line was added to help visualization of the correlations. Both panels showed a significant correlation at the $p < 0.05$ level.

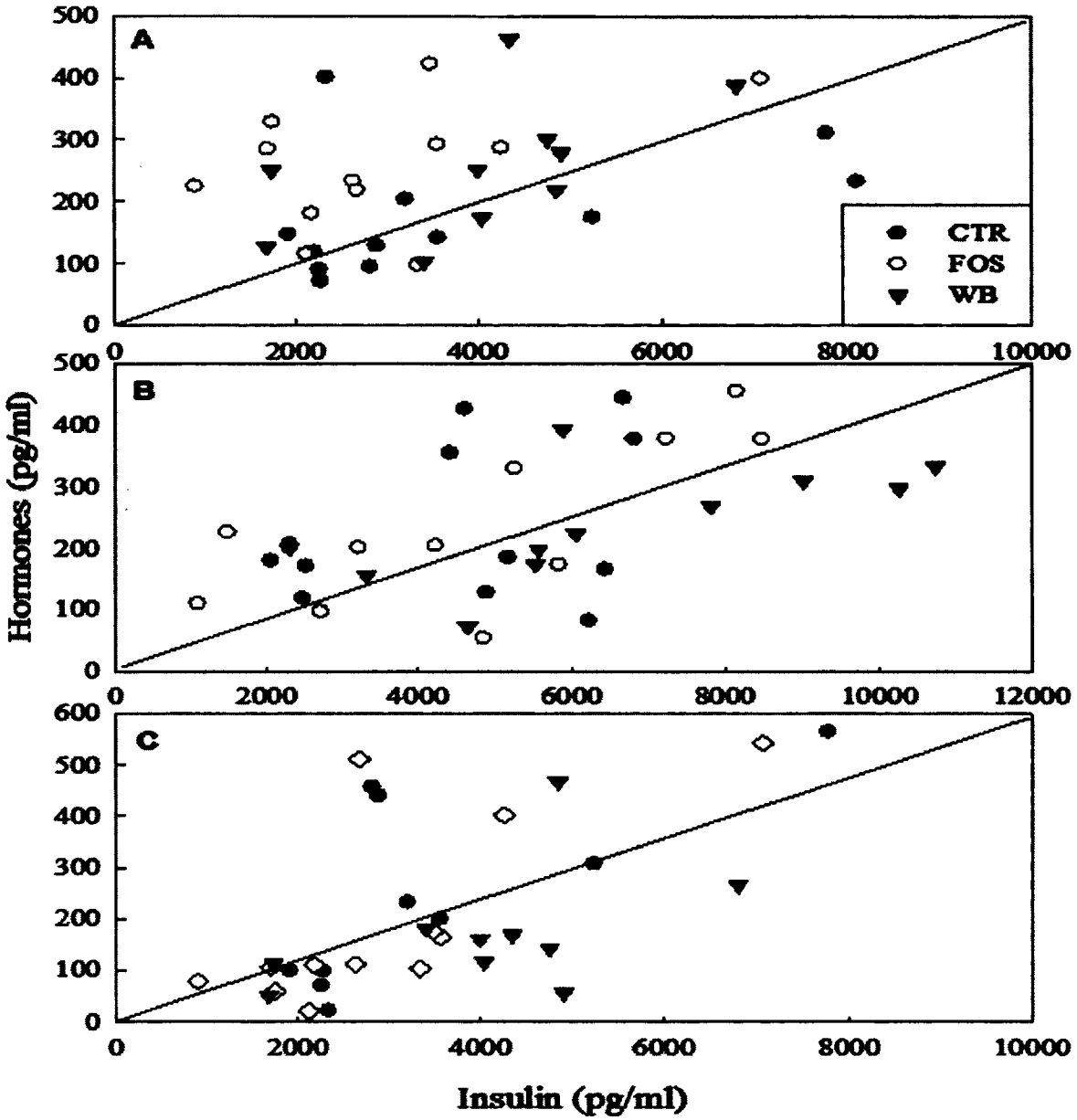


Figure 3.12: Relationship between levels of circulating active ghrelin or GLP-1 with insulin of F344 rats fed one of the three diets after water or glucose preload. The experimental treatment diets were control (black circles), FOS (white circles) and WB (inverted grey triangles). The preloads were either 2.5 mL water or 0.75 g glucose in 2.5 mL water. Serum hormone levels were determined by Luminex analyses. Panel A: correlation between level of active ghrelin and insulin after water preload; panel B: active ghrelin and insulin after glucose preload; and panel C: active GLP-1 and insulin after water preload. Values represent mean (n=12 per diet group). The data were analyzed using simple regression and both panels showed a significant correlation at the $p < 0.05$ level. Diagonal line was added to help visualization of the correlations.

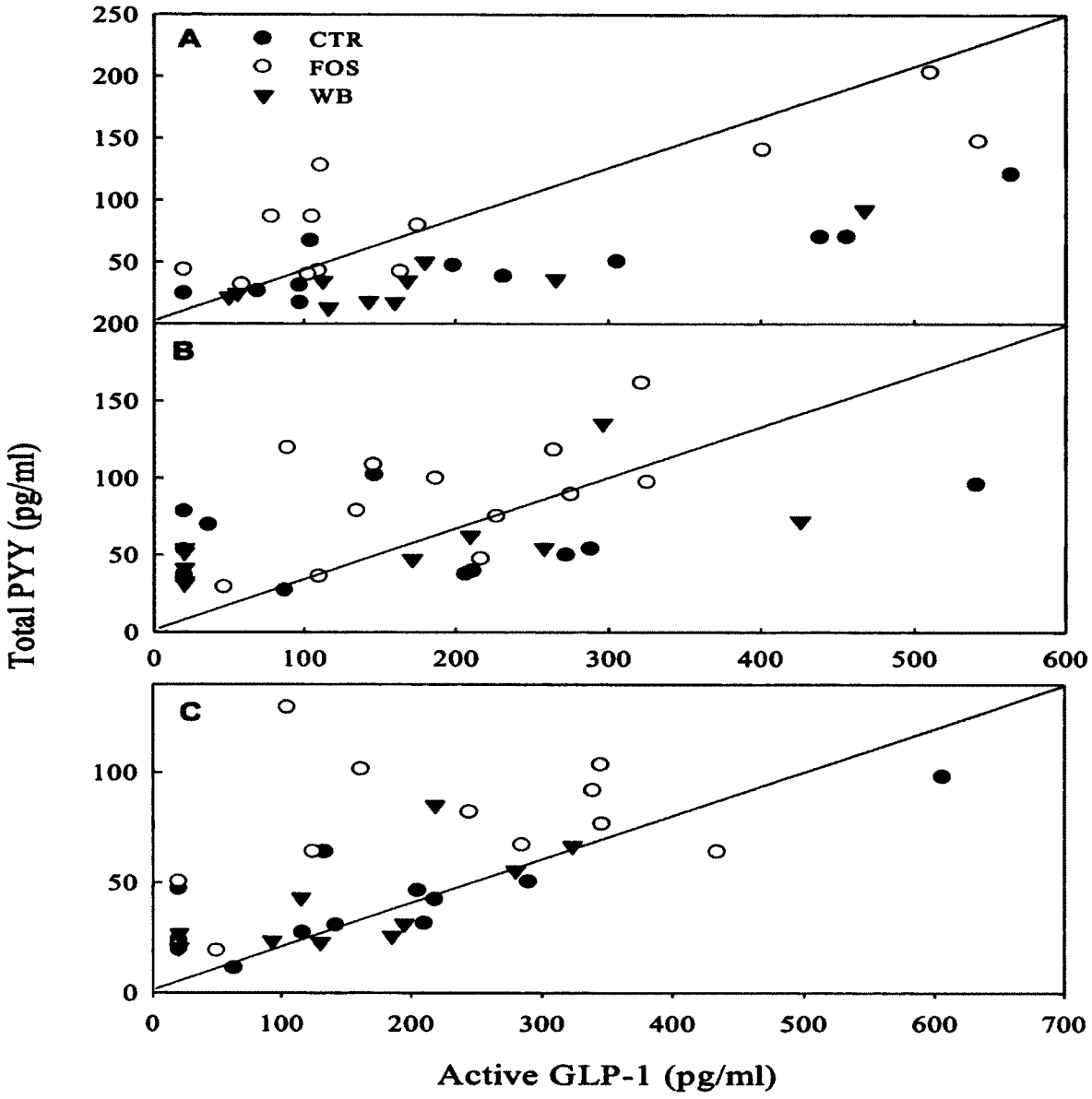


Figure 3.13: Relationship between levels of circulating total PYY and active GLP-1 after gavage in F344 rats fed one of the three diets. The experimental diets were control (black circles), FOS (white circles) and WB (inverted grey triangles). The preloads were 2.5 mL water, 0.75 g glucose or protein in 2.5 mL water. Levels of serum hormones were determined by Luminex analyses. Panel A: water preload; panel B: glucose preload; and panel C: protein preload. Values represent mean (n=12 per diet group). The data were analyzed using simple regression and all panels showed a significant correlation at the $p < 0.05$ level. Diagonal line was added to help visualization of the correlations.

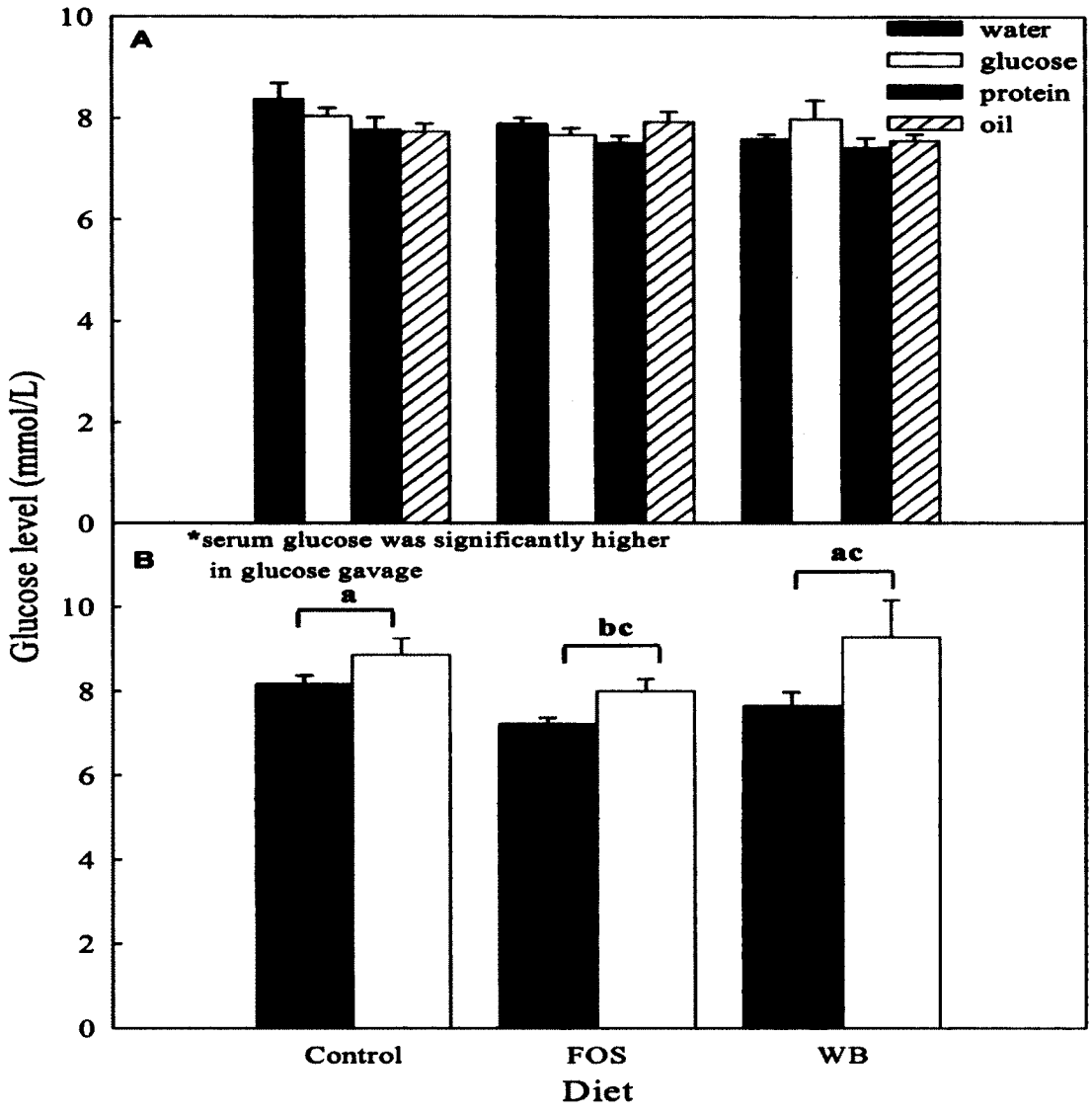


Figure 3.14: Serum glucose levels of F344 rats fed one of the three diets 30 min post nutrient preload. The experimental diets were control, FOS and WB. The nutrient preloads were 2.5 mL water (black bars), 0.75 g glucose (white bars) or protein (black bars with dots) in 2.5 mL water; and 0.8 mL oil (diagonal lined squares). Rats were gavage fed with either water or glucose before necropsy. Panel A: serum samples obtained from tail vein and panel B: serum samples obtained during necropsy. Values represent mean ± SEM (n=12 per preload). Data from panel A were analyzed using repeated measures ANOVA with diet and preload as factors. Panel B was analyzed by two-way ANOVA with diet and preload as categorical predictors; $p < 0.05$ was considered as significantly different. Diet groups sharing the same letter were not significantly different. * denotes glucose gavage was significantly different from water gavage.

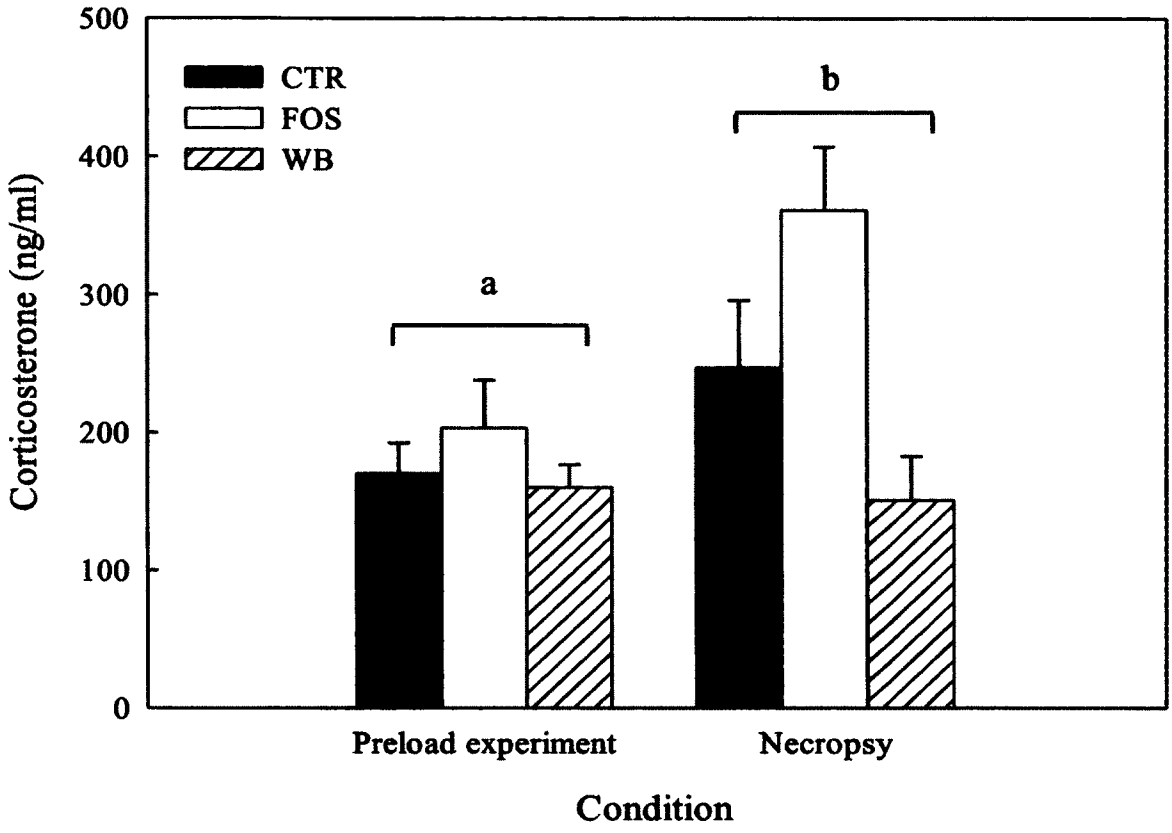


Figure 3.15: ELISA analysis of serum corticosterone levels in F344 rats fed one of three diets. Corticosterone was measured in rat serum obtained 30 min after water preload by the tail vein sampling or from torso blood after decapitation. The experimental diets were control (black bars), FOS (white bars) and WB (diagonally lined bars). Values represent mean ± SEM (n=6 per diet). Data were analyzed using two-way ANOVA with diet and condition as categorical predictors; $p < 0.05$ was considered as significantly different. Different letter denote values were significantly different between the two conditions.

Chapter 4

Results

Sprague Dawley rats

Physical measurements and long term food intake

At the start of the study, SD rats weighted around 200 g at 7 weeks old (Figure 4.1). Body weight gain was more rapid till week 4 and then slowed. A significant influence of diet on body weight or body composition was not observed. However, rats in the FOS group consistently gained less weight than CTR and WB, which approached statistical significance after week 2. Rats in the FOS group also had lower body fat mass than those in the CTR and WB groups, and this also approached significance after week 4 (Figure 4.2; $p=0.07$). Rats gained less weight as they grew older, and weight gain slowly decreased towards the end of study (Figure 4.3 A).

Daily energy consumptions were fairly constant within diet groups. Energy intake dropped significantly in all three groups ($p<0.001$) after week 5 and then remained constant until the end of study. Overall, rats in the FOS group consumed significantly less energy than WB ($p<0.005$). Energy consumption in the CTR group was not different from FOS or WB (Figure 4.3 B). Food efficiency was determined by dividing weekly weight gain by energy consumption. It was higher when rats were growing more rapidly and decreased as weight gain slowed towards the end of the study (Figure 4.3 C). There was no difference among the three diet groups.

The effect of macronutrient preloads on short term satiety

Figure 4.4 shows energy consumption after 30 min, 60 min, 120 min, 8 h (remaining dark cycle), and 10 h (daily total). Protein was the only preload that affected energy intake, lowering energy intake at 30, 60, and 120 min. There was no difference in energy intake among water, glucose and oil preloads up to 2 h post gavage. At 30 min

(Figure 4.4A), food consumption after water and oil preloads was significantly higher than protein ($p<0.001$). No diet effect was observed but rats fed the FOS diet consumed less energy than those fed WB and this approached statistical significance ($p=0.06$). At 60 min, and effect of diet and preload on energy intake was observed (Figure 4.4B). Energy consumption was the highest in rats fed the WB diet, and this was significantly higher than energy consumption in the rats fed the FOS diet ($p<0.05$). Energy consumption after the protein preload was significantly less than the other preloads ($p<0.001$). The effect of the protein preload was still visible at 120 min (Figure 4.4C); at this time energy intake in rats fed the protein preload was significantly than water ($p<0.05$). Although no significant diet effect was observed, energy consumption in rats fed the FOS diet was noticeably lower than CTR and WB. Overall, Figure 4.4 A-C showed that water, glucose and oil preloads had similar effects on short term energy consumption, whereas a protein preload suppressed energy intake.

After 120 min, rats were switched back to their experimental diets for the rest of dark cycle. A different trend was observed during this time (hour 2-10, Figure 4.4 D). Energy consumption was the highest after protein preload but there were no significant differences among water, glucose and protein. Energy consumption tended to be lower in rats fed the oil preload and it was significantly lower than protein ($p<0.005$). Measurement of total daily energy consumption post preload (Figure 4.4 E) showed that water, glucose and protein had similar effects on energy consumption. On the other hand, rats fed the oil preload consumed less energy than the rest of the preloads, and this was significant when compared to glucose ($p<0.05$) and approached significance when compared to water ($p=0.05$). Although the effect of the preload was evident throughout

the day, there was no diet or preload effect on daily total energy intake, when the energy of the preloads was included (Figure 4.4 F). Nevertheless, rats fed the FOS diet consumed less energy than WB on daily total energy but this was not significant (Figure 4.4 F, $p=0.06$). No diet X preload interaction was observed.

Real-time PCR analyses on mRNA hormone levels

Rats were gavage fed either 2.5 mL water or 0.75 g glucose in 2.5 mL water 30 minutes prior to sacrifice. All genes of interest were normalized to 2 housekeeping genes: HPRT and RPL10A.Water gavaged and CTR diet fed rats acted as control in the analysis and the values were normalized to 1.0.

Hypothalamus arcuate nucleus hormones

Figure 4.5A-D show that there was no significant effect of diet or preload on the mRNA levels of AgRP, POMC, CART, and NPY.

Lower gastrointestinal tract

Relative PGC and PYY levels were unaffected by either the preloads or diets in ileum (Figure 4.6A-B). There was an effect of both the diet and preload in the cecum. Feeding a glucose preload increased relative PGC mRNA levels to values approximately 1.8 times water preload levels ($p<0.05$). In addition, rats fed the FOS diet had almost 3 times higher PGC mRNA levels than rats fed the CTR or WB diet (Figure 4.6C, $P<0.005$). These diet and preload effects were also observed for PYY mRNA levels

(Figure 4.6D). PYY mRNA levels in rats fed the FOS diet were about 2.2 times those of the rats fed the control diet and 3 times those of the rats fed the WB diet ($p<0.001$). Rats fed glucose preloads had PYY values that were 1.5 times those of rats fed water preloads ($p<0.05$). The relative mRNA levels of PGC and PYY in rats fed water and preloads were very similar in the CTR and WB groups. The diet X glucose preload interaction approached significance, however, only the FOS group seemed to be further impacted by the glucose preload. As a result, glucose seemed to have an additive effect on both PGC and PYY mRNA level but to only in rats fed the FOS diet.

No significant effect of diet or preload was observed for either PGC or PYY mRNA levels in proximal and distal colon (Figure 4.6 E-H). However, the water preloaded WB group tended to have lower PGC and PYY mRNA levels than CTR or FOS group in the proximal colon (Figure 4.6 E-F). In the distal colon, rats fed the WB diet tended to have higher levels of both PGC and PYY mRNA after water preload than rats fed the CTR or FOS diets (Figure 4.6G-H).

Stomach

No effect of gavage or diet was observed on ghrelin mRNA levels.

Cecum size

Fermentation and diet affected the weight of the cecum and the cecal contents (Figure 4.8). Both the cecum size and cecal content were significantly higher in FOS-fed rats compared to those fed CTR or WB diets ($p<0.001$).

Distribution profile of relative mRNA in lower gastrointestinal tract

PGC (Figure 4.9A)

The relative PGC mRNA levels were: ileum > cecum > proximal colon > distal colon. The effect of diet and preload on the relative levels was not significant but some trends were apparent. In the ileum, it appeared that the mRNA levels in water gavaged FOS and WB fed rats were higher than CTR fed rats.

PYY (Figure 4.9B)

The relative PYY mRNA levels were: ileum > cecum > proximal colon > distal colon. Interestingly, even though cecum expressed lower PYY mRNA, FOS diet stimulated similar PYY expression in the cecum as the ileum. Rats fed the FOS diet after water preload stimulated higher PYY expression in the cecum than those fed CTR diet after water preload in the ileum. Furthermore, rats fed the FOS diet after glucose preload had the highest PYY expression in the lower GI tract.

Luminex analyses of circulating hormones in serum

The levels of active ghrelin were: water (control) >> oil >> glucose >> protein (all significantly different from each other; Figure 4.10A). Active ghrelin levels were: glucose (0.7 times control), protein (0.5 times control) and oil (0.8 times control). Levels of active ghrelin were unaffected by diet. Therefore, there was no preload X diet interactions.

The levels of active insulin were: glucose > protein > oil > water (Figure 4.10B). Rats fed the glucose and protein preloads had approximately 1.5 times higher insulin levels than water gavaged rats. Levels of insulin in rats fed oil preloads were not different from those fed water preloads. Levels of insulin were unaffected by diet. Therefore, there was no preload X diet interactions.

Levels of active GLP-1 were unaffected by preload or diet (Figure 4.10C).

The levels of circulating total PYY were the highest after oil preload: oil >> glucose > protein, water. Rats fed the oil preload had approximately 2 times the value in water gavaged rats ($p<0.001$; Figure 4.10D). PYY levels after water, glucose and protein preloads were not statistical significantly different. There was no diet effect and therefore, no preload X diet interaction was observed.

Correlations between hormones and energy consumption

Relationships between levels of circulating hormones and energy consumption at different time points were studied as well as the relationships between circulating hormones after the same preload. Only the relationships showing a significant correlation are presented.

None of the hormones were significantly correlated with energy consumption during the first two hours post preload. Active GLP-1 was strongly correlated with total PYY at 30 min post preload after water, glucose and protein preload (Figure 4.11 A-C,

respectively, $p<0.001$). No correlation was observed after the oil preload. The relationships seemed to be independent of treatment diet.

Serum glucose and corticosterone

The effect of macronutrient preloads on serum glucose levels was studied using two different samples: tail vein blood samples taken 30 minutes after each preload, and trunk blood collected during necropsy.

Different macronutrients had different effects on serum glucose levels (Figure 4.12). It was surprising to note that serum glucose levels were the same in rats fed water and glucose preloads, and were both significantly higher than protein or oil preloads (Figure 4.12A, $p<0.001$). Rats fed the FOS diet had significantly lower overall serum glucose level than CTR or WB ($p<0.05$). There was significant diet X preload interaction ($p<0.001$). More specifically, the rats fed the FOS diet and protein preload (abbreviated as FOS-protein) had significantly lower serum glucose level than CTR-water ($p<0.001$), FOS-water ($p<0.001$), WB-water ($p<0.001$) and WB-protein ($p<0.05$). Rats the FOS diet and oil preload (FOS-oil) had significantly lower serum glucose levels than CTR-water and CTR-oil ($p<0.01$), FOS-water ($p<0.001$), as well as WB-water ($p<0.001$) and WB-oil ($p<0.001$). Serum glucose was not changed in necropsy samples (Figure 4.12B). Rats fed the CTR diet had slightly higher serum glucose levels than those fed FOS and WB diets (by 1.1 times and 1.2 times control values, respectively; $p=0.06$ and $p<0.005$).

Corticosterone

Similar to the situation in F344 rats, it was decided to measure corticosterone levels in rats under the two conditions. Levels of corticosterone were not significantly different under the different conditions or among the diets (Figure 4.13). Corticosterone in rats fed the FOS diet tended to be higher WB (by 1.7 times that of rats fed WB diets but the effect only approached significance, $p=0.07$). Corticosterone levels in tail vein sampled rats were 1.2 times those of rats after sacrifice and this approached significance ($p=0.1$).

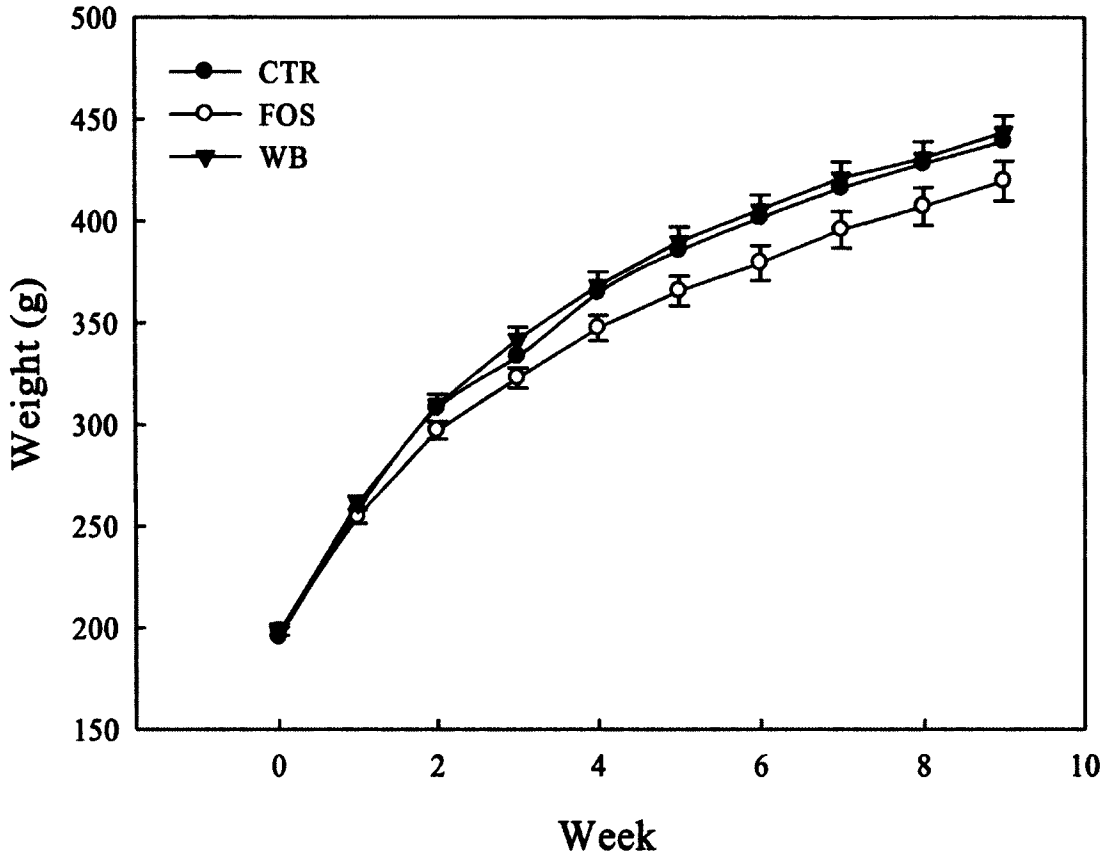


Figure 4.1: Weekly body weights of SD rats fed one of the three treatment diets. Three treatment diets were control (black circles), FOS (white circles) and WB (inverted grey triangles). Rats were 7 weeks old at week 0. Body weights were measured on Friday afternoons. Representative line and scatter plot showing mean values ± SEM (n=12 per diet group). Data were analyzed using one-way ANOVA; $p < 0.05$ was considered significantly different.

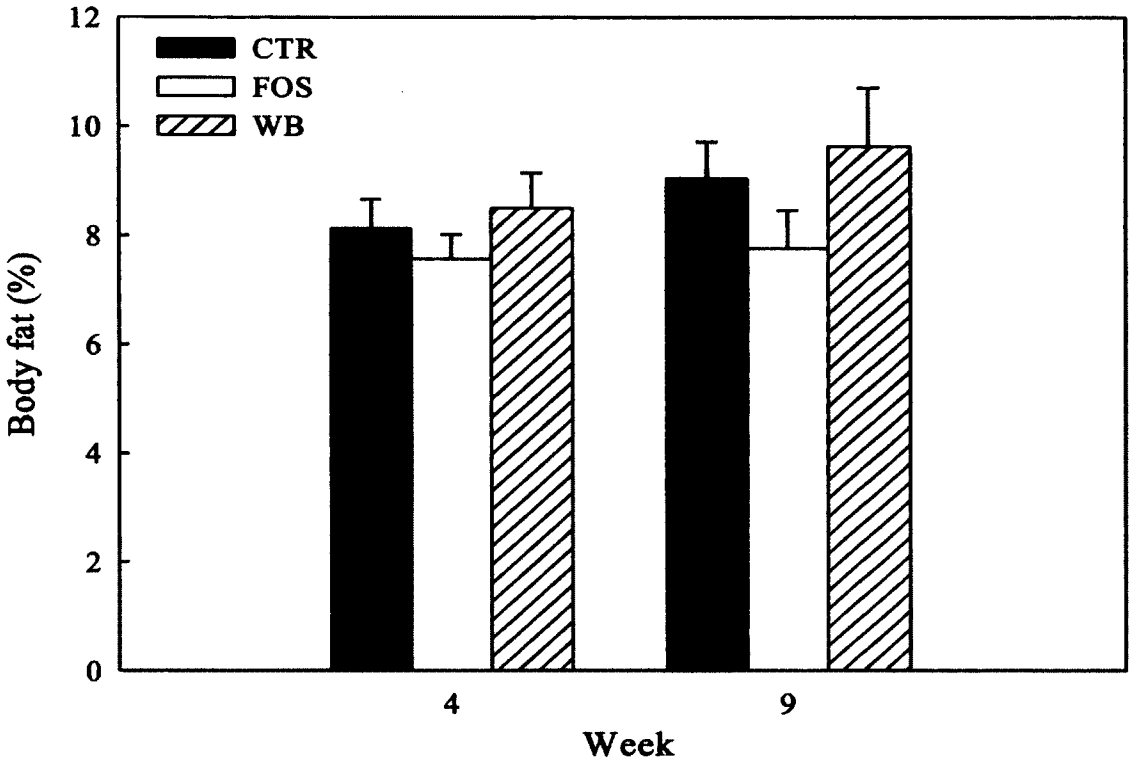


Figure 4.2: Body composition of SD rats fed one of the three treatment diets. The diets were control (black bars), FOS (white bars) and WB (lined bars). Percentage body fat was determined based on the weight of fat mass analyzed by EchoMRI instrument over the body weight. Body composition was done immediately after the body weight measurements; two time point of body composition measurements were before the nutrient preload experiment and before necropsy. Representative histograms showing mean values ± SEM (n=12 per diet group). Data were analyzed using one-way ANOVA; No significant differences were observed at the $p < 0.05$ level.

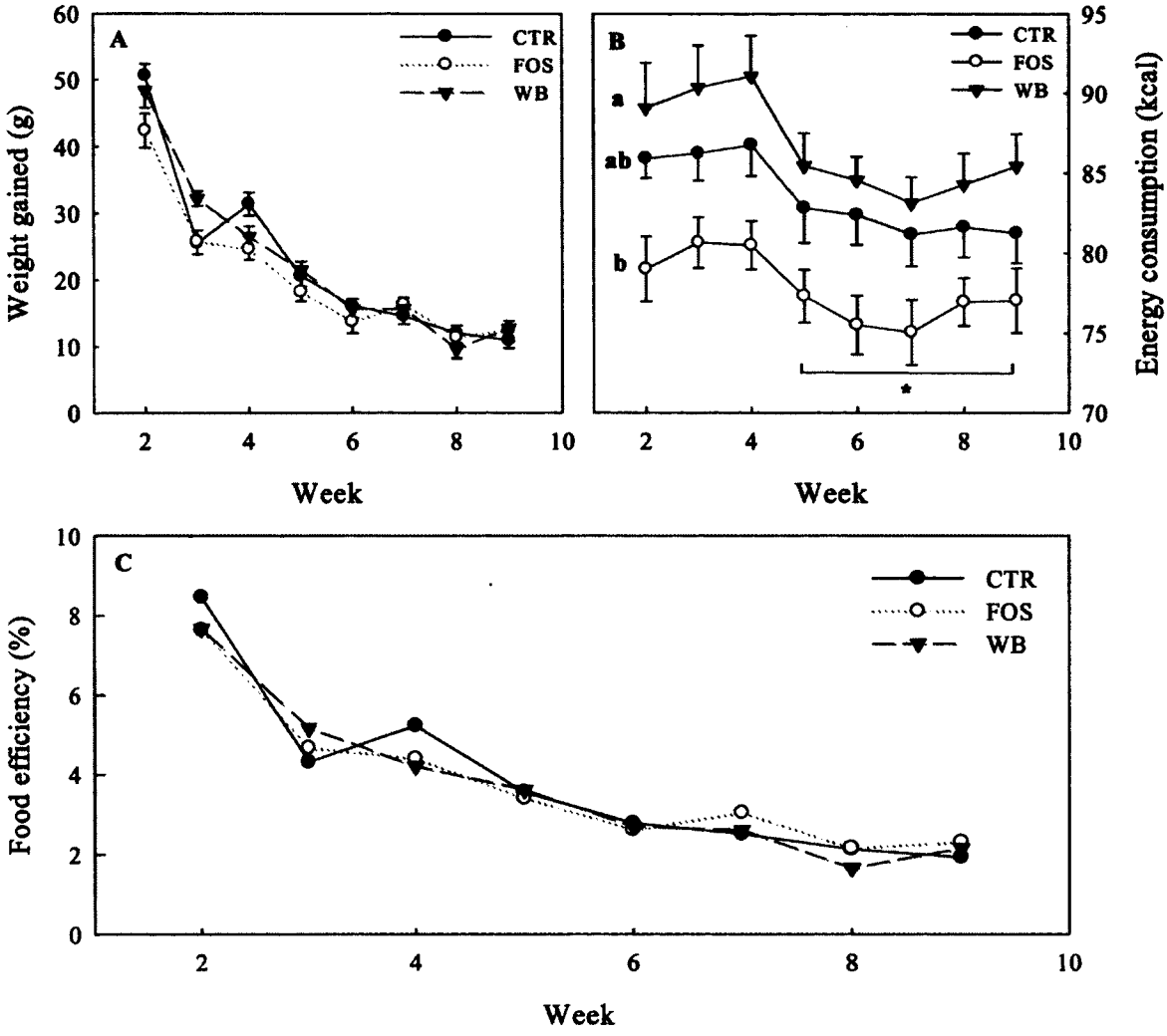
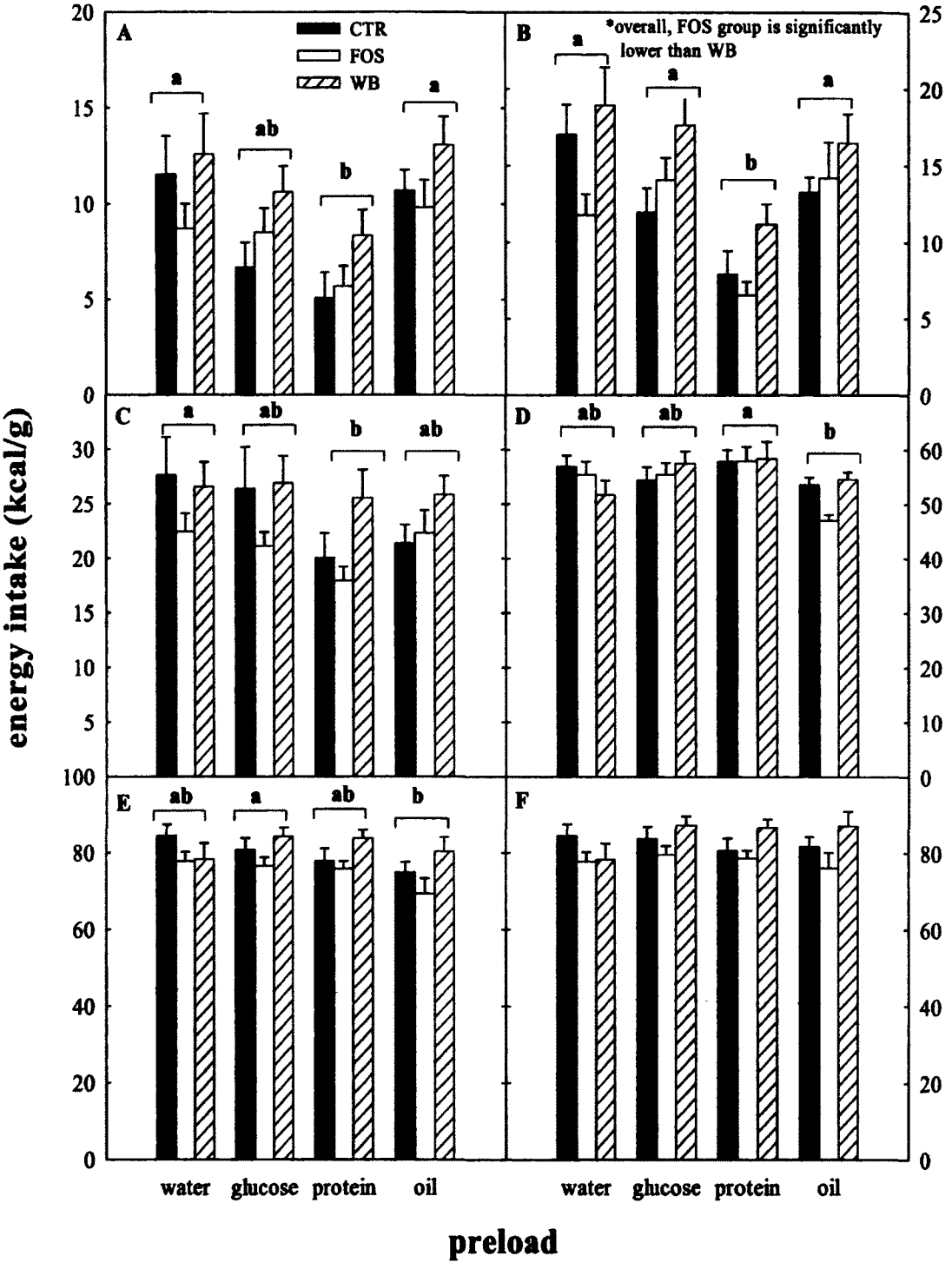


Figure 4.3: Food efficiency of SD rats fed one of the three treatment diets throughout the study. Three treatment diets were control (black circles), FOS (white circles) and WB (inverted grey triangles). Food efficiency was determined by weekly body weight gained over weekly energy consumed (weight gained/kcal). Panel A: average weekly weight gained; panel B: average daily energy consumption per week; and panel C: average weekly food efficiency. Representative line and scatter plot showing mean values ± SEM (n=12 per diet group). Week 1 data was excluded due to insufficient food intake measurements. Data were analyzed using repeated measures ANOVA; $p < 0.05$ was considered as significantly different. Diet groups sharing the same letter were not significantly different. Asterisk denotes that week 5 to 9 was significantly different from week 2 to 4.

Figure 4.4: Energy consumption of SD rats at various time points after gavaged fed with one of the four macronutrient preloads 30 minutes prior to the onset of dark cycle. The four nutrient preloads were 2.5mL water, 0.75g of glucose or protein in 2.5mL water and 0.8mL oil. Three treatment diets were control (black circles), FOS (white circles) and WB (inverted grey triangles). All rats were fed with control diet for the first 2 hours to eliminate possible confounders that might affect food intake. Energy consumption was calculated by multiplying amount of food intake measured (grams) by the caloric density of the diet (3.7kcal/g). Panels A-E were data measured post preload whereas panel F was the daily total including energy density of the preloads. Panel A: First half an hour; panel B: first hour; panel C: first 2 hours; panel D: from 2-10 hours (treatment diets); panel E: daily total without energy consumed from preload; and panel F: daily total including energy density obtained from preload. Representative histograms showing mean values ± SEM (n=12 per diet group). Data were analyzed using repeated measures ANOVA with diet as categorical predictors and 4 levels of within subject factors; $p < 0.05$ was considered as significantly different. Diet groups sharing the same letter were not significantly different. * denotes the FOS group consumed significantly less energy compared to the WB group.

Figure 4.4


A
B
C
D
E
F
CTR
FOS
WB
*overall, FOS group is significantly lower than WB
energy intake (kcal/g)
water
glucose
protein
oil
preload

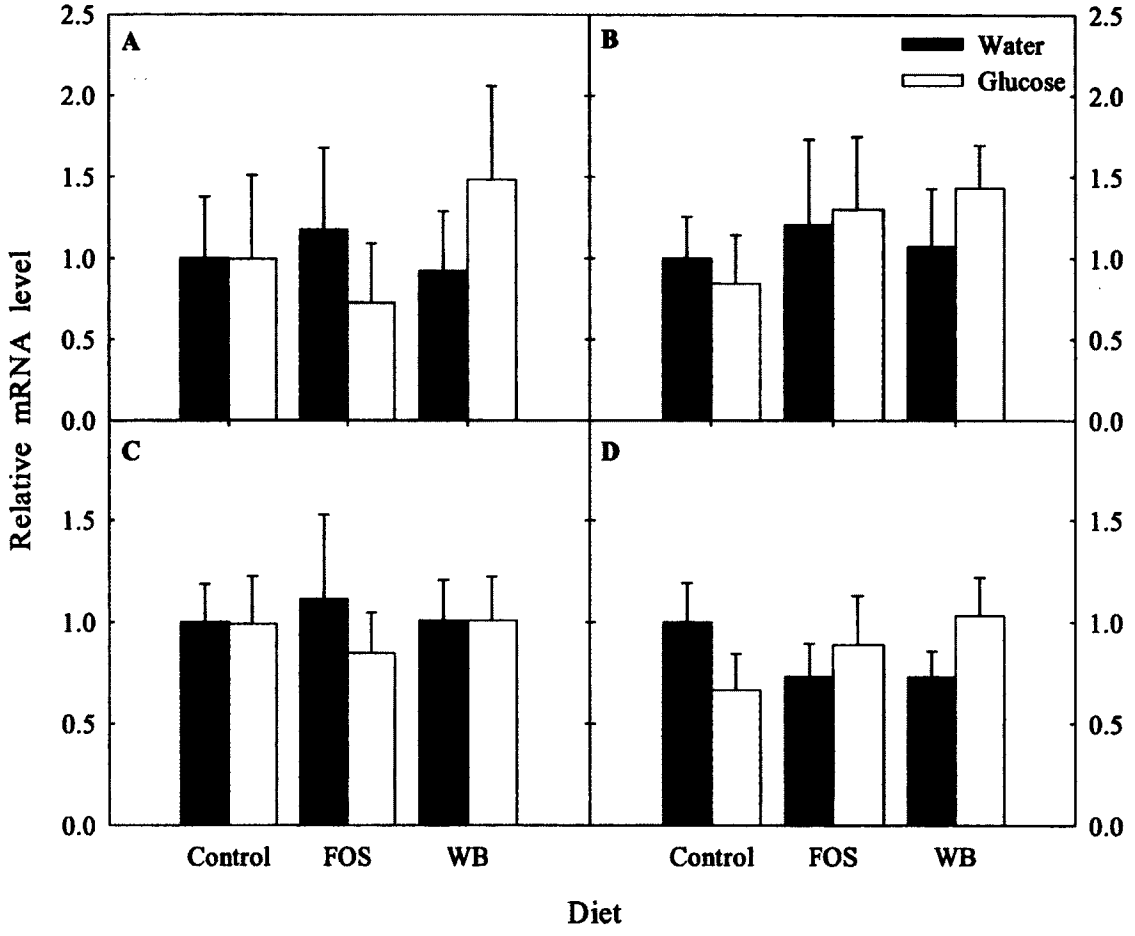
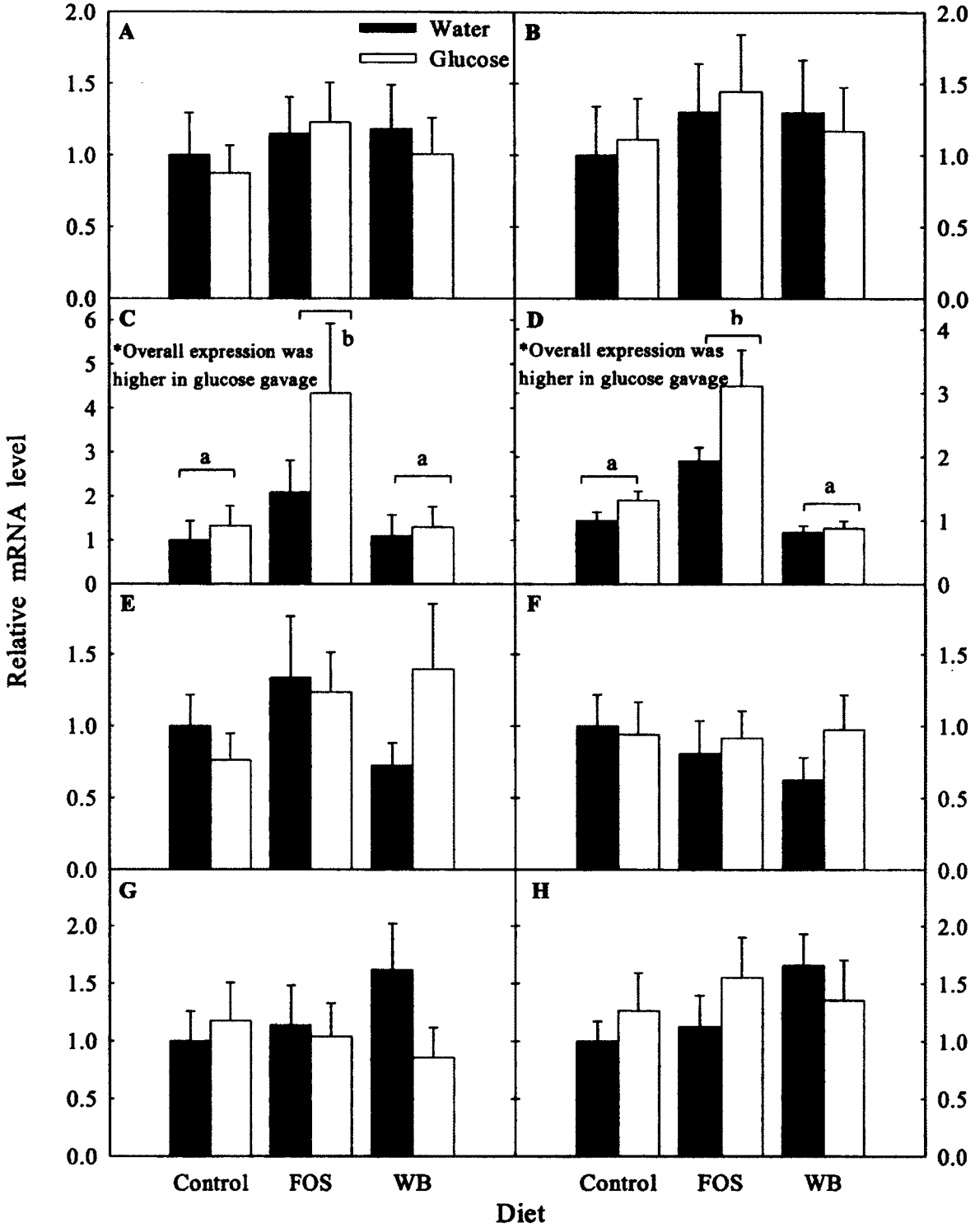


Figure 4.5: Real-time PCR analysis of mRNA hormone levels in hypothalamus arcuate nucleus (ARC) of SD rats fed one of the three diets measured after necropsy. Rats were gavage fed preload contained either 2.5mL water (closed squares) or 0.75g of glucose in 2.5mL water (open squares) 30 minutes prior to sacrifice. Panel A: AgRP in ARC; panel B: POMC in ARC; panel C: CART in ARC; and panel D: NPY in ARC. The values for water gavaged control fed rats were normalized to 1.0. All other values are relative to water gavaged control fed rats. Representative histograms showing normalized fold differences ± SEM (n=6 per preload, n=12 per diet group). Data were analyzed using two-way ANOVA with type of diet and preload as categorical predictors; $p < 0.05$ was considered as significantly different.

Figure 4.6: Real-time PCR analysis of mRNA hormone levels in the lower gastrointestinal tract of SD rats fed one of the three diets measured after necropsy. Rats were gavage fed preload containing either 2.5mL water (closed squares) or 0.75g of glucose in 2.5mL water (open squares) 30 minutes prior to sacrifice. Panel A: PGC in ileum; panel B: PYY in ileum; panel C: PGC in cecum; panel D: PYY in cecum; panel E: PGC in proximal colon; panel F: PYY in proximal colon; panel G: PGC in distal colon; and panel H: PYY in distal colon. The values for water gavaged control fed rats were normalized to 1.0. All other values are relative to water gavaged control fed rats. Representative histograms showing normalized fold differences ± SEM (n=6 per preload, n=12 per diet group). Data were analyzed using two-way ANOVA with diet and preload as categorical predictors; $p < 0.05$ was considered as significantly different.

Figure 4.6


A
Water
Glucose
B
C
*Overall expression was higher in glucose gavage
a
b
a
D
*Overall expression was higher in glucose gavage
b
a
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E
F
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Relative mRNA level
Control
FOS
WB
Control
FOS
WB
Diet

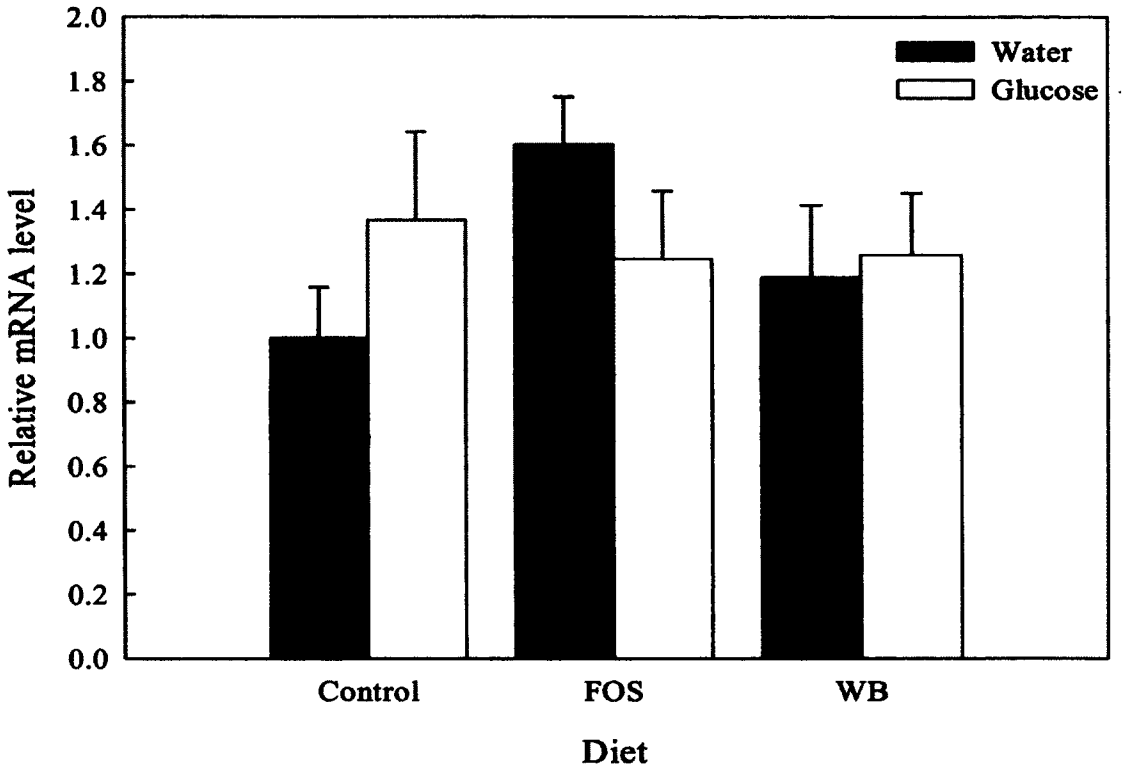

Figure 4.7: Real-time PCR analysis of mRNA levels of ghrelin in the stomach of SD rats fed one of the three diets measured after necropsy. Rats were gavage fed preload contained either 2.5 mL water (closed squares) or 0.75g of glucose in 2.5 mL water (open squares) 30 minutes prior to sacrifice. The values for water gavaged control fed rats were normalized to 1.0. All other values are relative to water gavaged control fed rats. Representative histograms showing normalized fold differences ± SEM (n=6 per preload, n=12 per diet group). Data were analyzed using two-way ANOVA with diet and preload as categorical predictors; $p < 0.05$ was considered as significantly different.

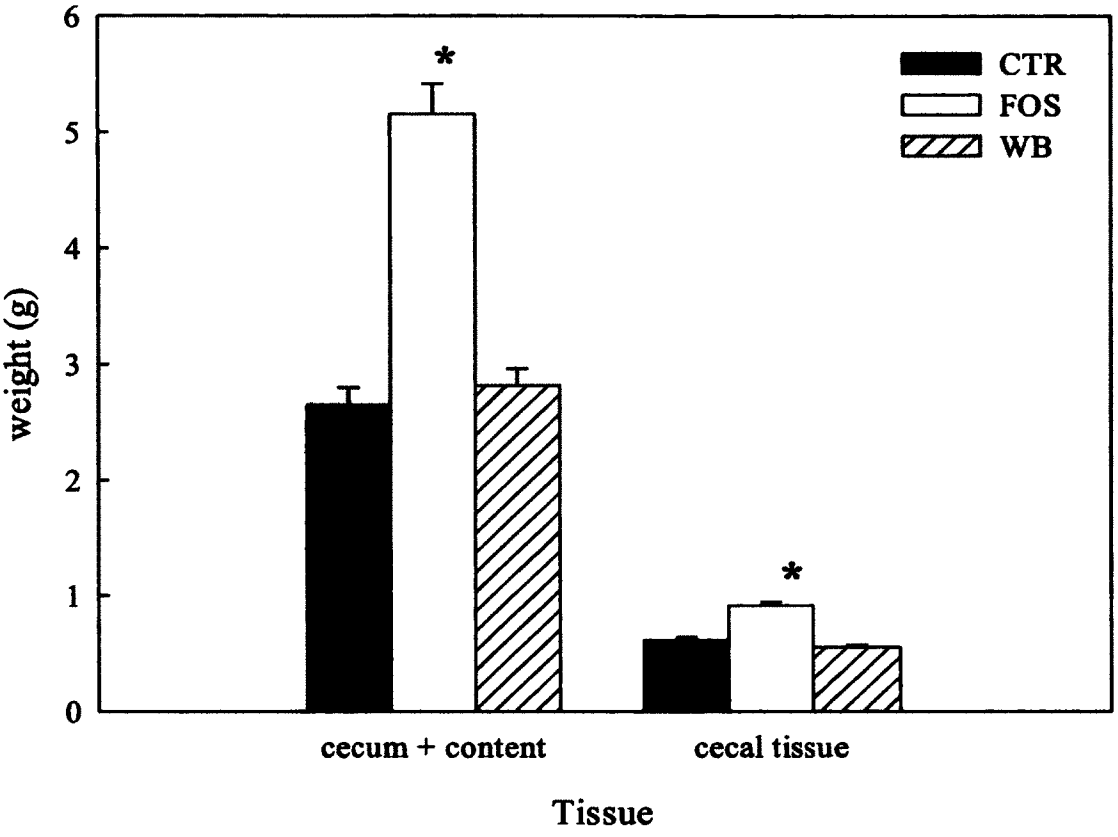

Figure 4.8: Cecal tissue sizes of SD rat fed one of the three diets weighted during necropsy. Three treatment diets were control (closed bars), FOS (opened bars) and WB (lined bars). Representative histograms showing mean values ± SEM (n=12 per diet). Data were analyzed using one-way ANOVA; $p < 0.05$ was considered as significantly different. Asterisks denote that FOS group had significantly different compared to the CTR or WB group with or without cecal contents.

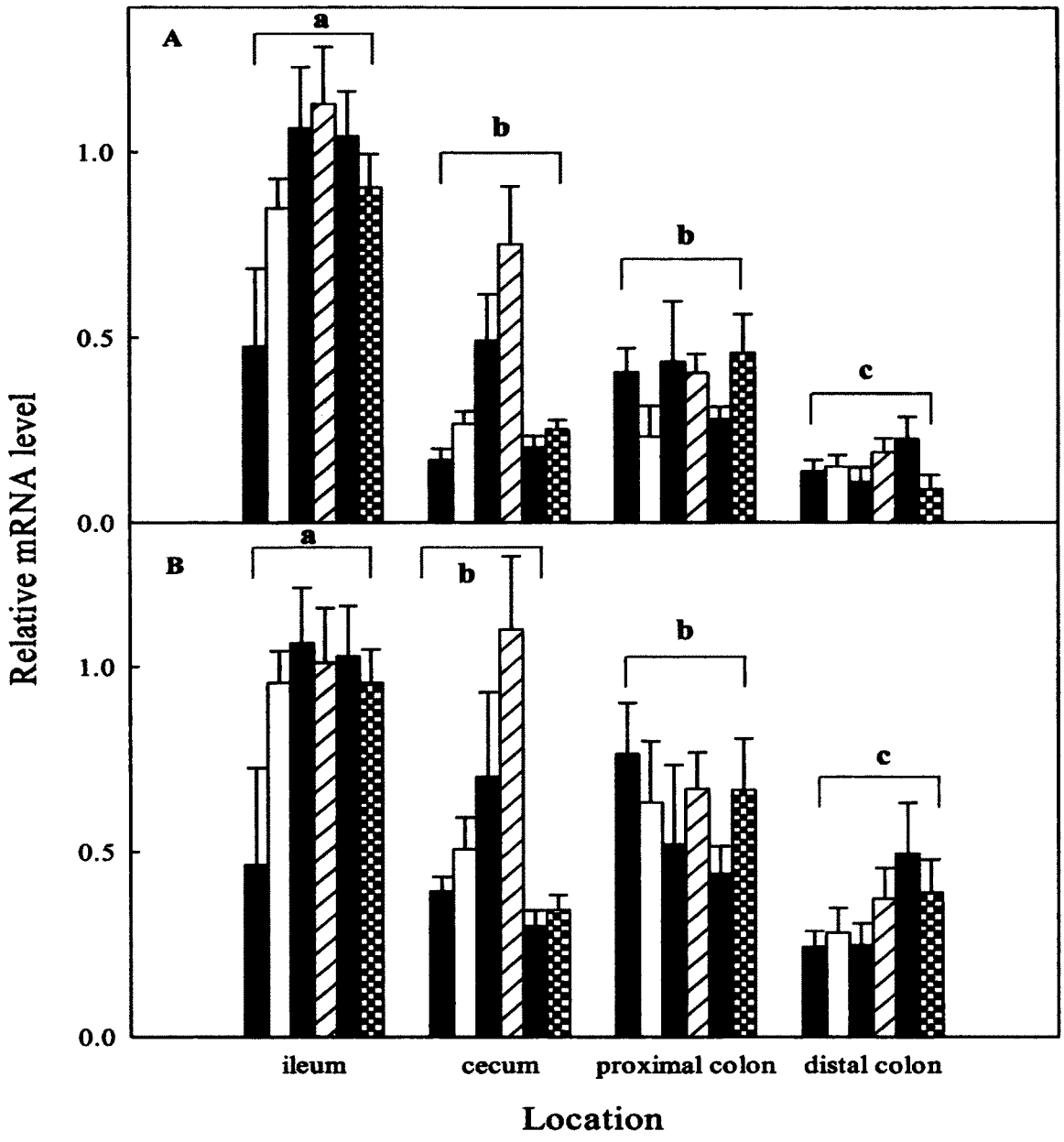

Figure 4.9: Distribution of mRNA hormone levels analyzed by real-time PCR in the lower gastrointestinal tract of SD rats fed one of the three diets measured after necropsy. Rats were gavage fed preload contained either 2.5mL water (gray bars) or 0.75g of glucose in 2.5mL water (white bars) 30 minutes prior to sacrifice. The treatment diets were: control (empty bars), FOS (lined bars) and WB (checker bars). Panel A: distribution of PGC and panel B: distribution of PYY. Representative histograms showing mean values of relative expression across tissues normalized to housekeeping genes ± SEM (n=6 per preload, n=12 per diet group). Tissues sharing the same letter were not significantly different.

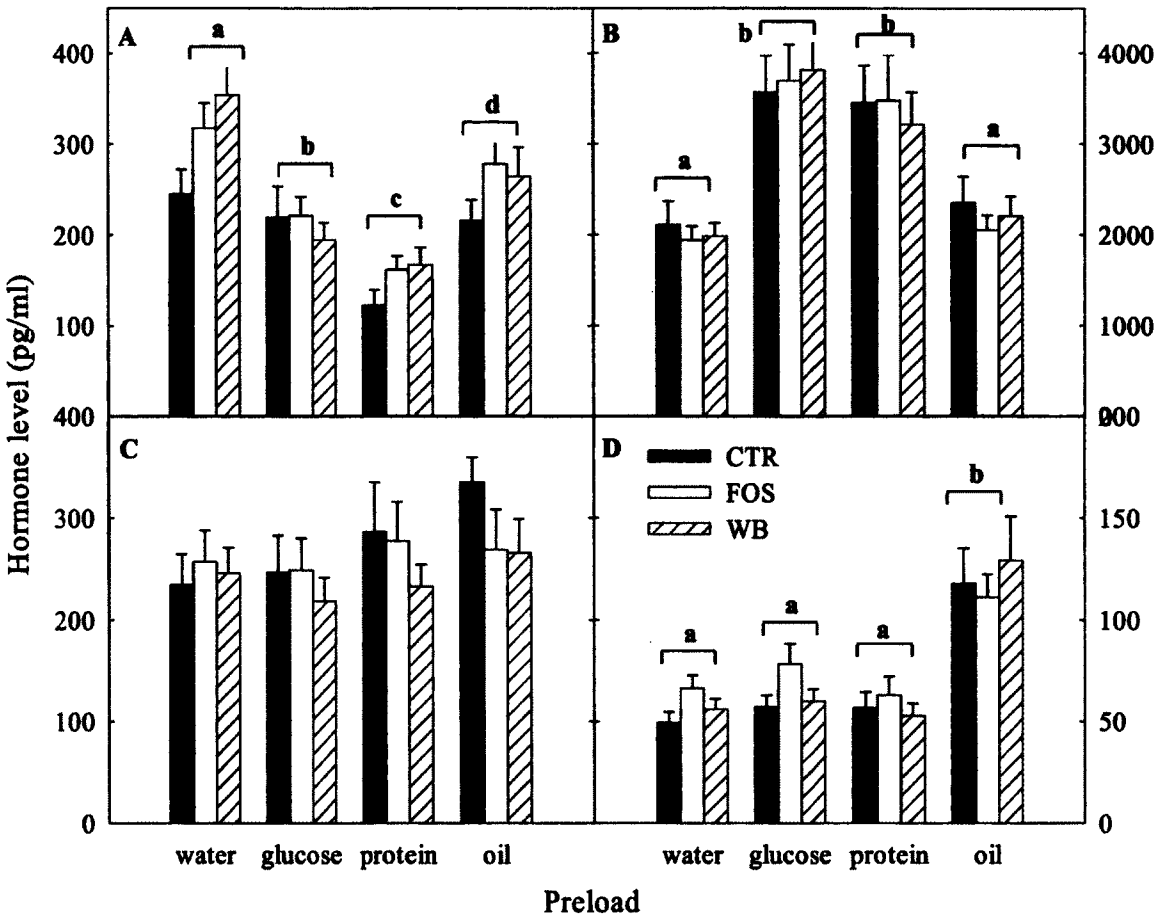

Figure 4.10: Luminex analysis of serum hormone levels of SD rats fed one of the three diets measured 30 minutes after each gavaged fed nutrient preload. Three treatment diets were control (closed bars), FOS (opened bars) and WB (lined bars). The four nutrient preloads were 2.5mL water, 0.75g of glucose or protein in 2.5mL water and 0.8mL oil. Panel A: active ghrelin; panel B: insulin; panel C: active GLP-1 and panel D: total PYY. Representative histograms showing mean values ± SEM (n=12 per diet group). Data were analyzed using repeated measures ANOVA with diet as categorical predictors and 4 levels of within subject factors; $p < 0.05$ was considered as significantly different. Only panels with significant differences observed were labeled with letters or asterisk. Diet groups sharing the same letter were not significantly different.

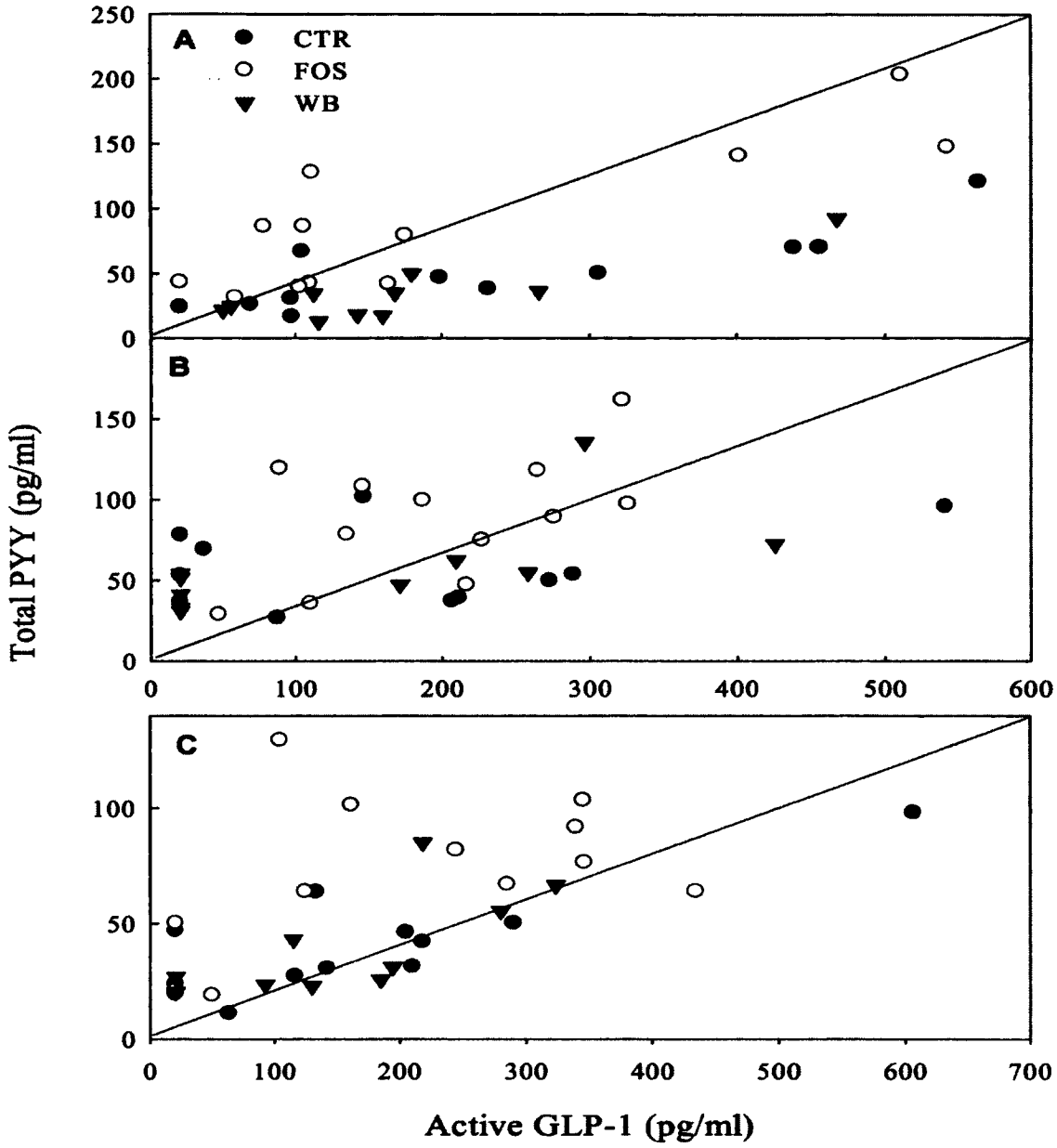

Figure 4.11: Relationship between level of circulating total PYY and active GLP-1 after three of the four preloads of SD rats fed one of the three diets. Three treatment diets were control (closed circles), FOS (white circles) and WB (inverted grey triangles). The preloads were 2.5mL water, 0.75g glucose or protein in 2.5mL water. Level of serum hormones were determined 0.5 h after gavage by Luminex on separate days. Panel A: water preload; panel B: glucose preload; and panel C: protein preload. Representative scatter plot showing mean values (n=12 per diet group). Data were analyzed using simple regression and all panels showed significant correlation; relationship with $p < 0.05$ was considered significantly.

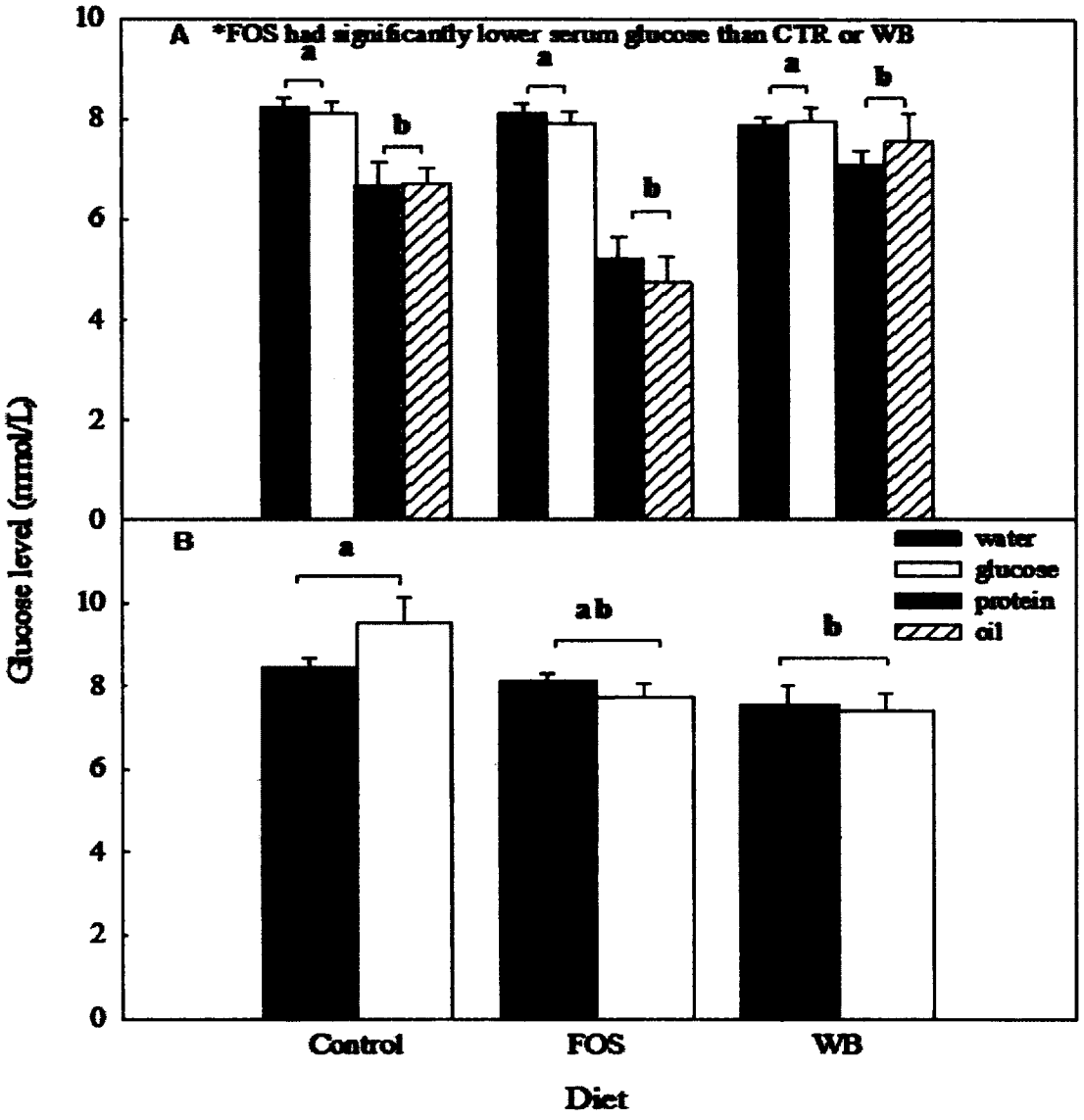

Figure 4.12: Serum glucose levels of F344 rats fed one of the three diets 30 minutes after nutrient preload. Three treatment diets were control, FOS and WB. The four nutrient preloads were 2.5mL water (closed squares), 0.75g of glucose (open squares) or protein (closed squares with dots) in 2.5mL water and 0.8mL oil (lined squares). Rats were gavaged fed with either water or glucose before necropsy. Panel A: serum samples taken during nutrient preload experiment and panel B: serum samples obtained during necropsy. Representative histograms showing mean values ± SEM (n=12 per preload). Data from panel A were analyzed using repeated measures ANOVA with diet as categorical predictors and 4 levels of within subject factors. Panel B was analyzed by two-way ANOVA with diet and preload as categorical predictors; $p < 0.05$ was considered as significantly different. Diet groups sharing the same letter were not significantly different. Asterisks denote that FOS group had significantly.

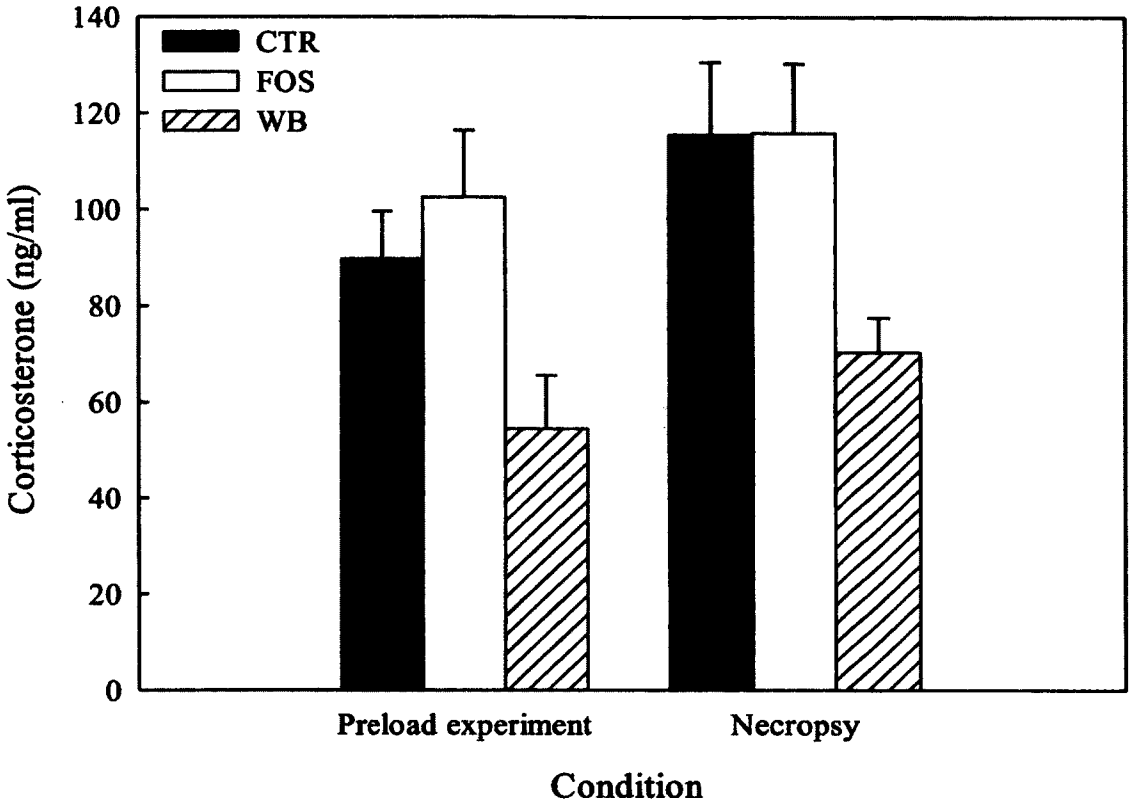

Figure 4.13: ELISA analysis on levels of serum corticosterone of SD rats fed one of the three diets. Corticosterone was measured in rat serum obtained 30 min after water preload by the tail vein sampling or from torso blood after decapitation. The experimental diets were control (black bars), FOS (white bars) and WB (lined bars). Values represent mean ± SEM (n=6 per diet). Data were analyzed using two-way ANOVA with diet and condition as categorical predictors; $p < 0.05$ was considered as significantly different. Different letter denote values were significantly different between the two conditions.

Chapter 5

Discussion

Among humans, the prevalence of overweight and obesity is increasing both nationally and worldwide. It is a growing concern as it increases the risk of health problems. A study done in the USA showed that increased energy intake appears to the major driver of the obesity epidemic [3]. For humans, the desire to eat is not only driven by survival needs but also by factors such as lifestyle and social events. Thus, the amount of food consumed once the meal is initiated becomes a key physiological control mechanism to protect against weight gain. As a result, diet modification as a weight management tool has gained great attention over the years. Investigations of macronutrient influence on food intake and satiety has shown that protein is the most satiating macronutrient and it has been shown to reduce meal size [127].

Dietary fibre has also been linked to weight management. Long term observational studies have consistently shown that DF intake is inversely related to weight gain [112,114,128,129]. Many claim that these beneficial effects of DF make them strong candidates for use as weight management tools. Food intake and weight gain are controlled by a complicated system that involves many biological regulatory mechanisms, including satiety signals associated with gut hormone action. However, scientific evidence is lacking to demonstrate the relationship among DF fermentation, macronutrient effect, and food intake over both the short and long term. Therefore the primary aim of this study was to study the interactions among DF, macronutrient, and hormonal responses in order to further understand their influence on food intake and weight gain. Two different types of DF were used to investigate potential differences in the effect of DF based on their fermentation rates. Two different rat models were used to identify whether the relationships investigated are conserved within species or strain

dependent. Rats were used in this study because of the similar anatomical characteristics and physiological responses to humans. In addition, rat studies form a large database, which is useful for comparative purposes [130]. Some of the other reasons for choosing rats as the animal model include: 1) Rats have high compliance in adhering to diet of choice, 2) By using rats, the studies can investigate mechanisms and regulators of interest in a controlled setting free from possible confounding factors, such as a large variability in human characteristics or environmental conditions, 3) It is relative easy to extract rat tissues for measurement of hormones, 4) Rats have been previously used in this field of work and studies have indicated that there is an effect of hormones, fermentation, and macronutrients on satiety in rats, 5) Data interpretation of human studies is more difficult because it is challenging to compare across studies that used different amounts and types of DF and macronutrient composition.

General comparison of macronutrient preloads on food intake

There are several studies in the literature comparing macronutrient effects in rats and they all differ in some respect. One study showed that glucose had no effect during the first two hours of feeding while corn oil and protein significantly lowered food intake in Wistar rats [131]. Another study indicated that all three types of preload significantly lowered the equivalent amount of food intake in lean Zucker rats and that food intake is affected primarily by calorie content [132] whereas Burggraf et al. demonstrated that at equal caloric content, glucose is more effective than fat in reducing short term food intake in Lewis rats [133]. Maggio et al. also suggested that protein and glucose had similar effects on food intake and were both more potent than corn oil in fatty rats [132]. It is

apparent that rat strains may differ in their physiological responses and this may influence the response to macronutrients. A major challenge when comparing across studies is that the experimental conditions (including rat models and preload contents) can differ significantly. In addition, there are problems with experimental details. For example, the calorie content was not reported in the Wistar rat study [131] and both the glucose and protein preloads were given at a higher energy density with larger preload volumes in the Zucker rat study. Different caloric content as well as volume of the preload have been shown to lead to a different pattern of food suppression [134]. The food intake response to macronutrients can also be rat model dependent, as was observed in our study. Differences in the source of macronutrient preload used in a study can also have a huge impact on the outcome. Despite these difficulties, it is generally accepted that protein is the most satiating macronutrient of the three. I observed that the three macronutrients had different effects on short term satiety and hormone responses.

Macronutrient preload effects: Protein

Consistent with previous studies [127], our data showed that that protein was associated with the highest satiety and energy compensation in both rat models as measured by food intake over the first 2 hours. However the effect of whey protein differed between strains with SD rats showing a compensation of 150% and F344 rats showing a compensation of 300% (Please see Appendix I for the equation to calculate energy compensation). This data suggests that the SD rat may have a more blunted response to a nutrient preload. Other published studies with a similar design using either

whey protein or casein have also shown that protein effectively reduced subsequent food intake even though energy compensation was not reported [131,132,135].

In the present study, the protein suppression of food intake observed during the first 2 hours did not translate into a prolonged inhibition of energy intake throughout the day. Thus, from 2-10 hours, the time period where food digestion in the stomach was completed and passed down to the lower GI tract (approximately 6 hours to reach the cecum), rats fed the protein preload consumed more food than all other preloads. Therefore, the effect of whey protein on satiety ended relatively quickly and the rats compensated for the loss of energy intake during the first two hours. On the other hand, study has shown that ^{13}C-labelled leucine from casein persisted in the plasma up to 7 hours post ingestion whereas more rapidly digested proteins, like whey protein exhibited a much higher leucine peak but values returned to normal by 4 hours. Although insulin responds differently to different amino acids, differences in plasma leucine concentrations were not the result of differences in plasma insulin, which stimulate gluconeogenesis [136,137]. It appears that the main determinant of leucine metabolism is substrate availability rather than insulin response [138]. In rats, circulating amino acids are largely unaffected by either high protein (casein) or high carbohydrate (starch) but portal vein concentrations of amino acids after 4 hours were higher on the high protein diet[139] showing that the liver was the major site of amino acid clearance and gluconeogenesis. These results show that, although circulating amino acids may not be cleared rapidly, they are probably only transiently raised and affect gut hormone release during digestion only.

In agreement with previously published studies [9,16,31,48,56], results from this study showed that protein significantly increased insulin release while suppressing ghrelin levels but did not affect PYY and GLP-1 secretion. Because protein is related to insulin and ghrelin, its anorexic effect is acute. Both ghrelin and insulin are closely linked to neuropeptide production in the ARC. Even though PYY and GLP-1 are important regulators of satiety and were unaffected by protein, our results suggest that PYY and GLP-1 are not greatly involved in protein mediated satiety. In other studies, rats fed a high protein diet had lower NPY mRNA expression and higher POMC mRNA expression in both *ad libitum* fed and 12 hour fasted state [140]. NPY stimulated food intake and POMC inhibited food intake [141]. Both acute (2 days) and chronic (21 days) treatments of rats fed a modified diet with high protein content led to reduced food intake and significantly higher numbers of c-Fos positive neurons in the nucleus of the solitary tract (NTS). The c-Fos neurons are often used as an indirect marker of neural activity and activation of neural activity in the NTS has been associated with satiety [135]. Increased α-MSH neurons that occur after high protein diets indicate the presence of higher levels of POMC neurons and activation of the melanocortin pathway in the ARC, which would be consistent with a decrease in food intake [135]. These results suggest that protein may either directly induce changes in neuropeptide activities or act indirectly through the action of insulin and ghrelin levels.

The mTOR is an intracellular signaling molecule that is sensitive to amino acids and is expressed by neuropeptide producing neurons. Activation of mTOR in the hypothalamus has been linked to acute food suppression following a high protein diet or leucine supplementation [142]. Furthermore, studies suggest that the effect of a protein diet

on food intake is largely dependent on the amount of leucine since leucine doses directly correlate with the reduction in food intake. Both diets high in protein and leucine treatment (that reduced food intake, weight and fat mass gain) were accompanied by mTOR activations in the ARC, higher POMC mRNA expression, and reduced NPY and AgRP mRNA expression [140,143]. The effect on food intake inhibition was completely reversed when mTOR activation was prevented [140]. Taken together, hypothalamic satiety control also seemed to be dependent on mTOR signaling. This suggests that amino acid composition in the protein diet influences neuropeptide production and is an important factor in controlling satiety.

A study found that diets high in protein content led to energy intake reduction by factors other than a higher energy expenditure since the major daily energy expenditure component of the resting metabolic rate was unaffected by protein consumption. Thus, the proposed mechanism of protein action appeared to be the ability to increase satiety by suppressing appetite [8]. As a result, satiety may be largely regulated by hormonal changes in response to food intake. Therefore, the acute satiety response associated with protein consumption may be mediated by its ability to suppress ghrelin, stimulate insulin and the direct involvement with neuropeptides rather than acting through gut hormones. This has also been observed in human studies where hunger rating was lower after a high protein meal by suppressing active ghrelin with low active GLP-1 or total PYY stimulation [144].

Macronutrient preload effects: Oil

It is generally accepted that fat exerts the weakest effect on satiety when compared to carbohydrate or protein because overeating fat attenuates responsiveness of the hormones that regulate feeding behaviour, such as insulin [145] and melanocortin peptides [146]. However, as mentioned earlier, preload experiments have shown mixed results on the effect of oil on food intake. In our study, the effect of oil preload differed between strains with SD rats showing a compensation of only 35% and F344 rats showing a compensation of 100%.This suggests that oil either promoted or had no effect on food intake over the first 2 hours (100% energy compensation means no difference in total energy intake between nutrient preload and control, less than 100% means higher energy consumption than control). A study showed that SD rats consumed more food after an isocaloric preload containing a higher fat content over the first 2 hours, which is consistent with our findings of SD rats. The higher food intake was associated with increased circulating level of triglycerides [147].

Despite the lack of effect on satiety after oil preload during 0-2 hours, both rat strains consumed less food than all other preloads from 2-10 hours. This led to a near significant reduction in daily total energy intake for SD rats and a significantly lower daily total energy intake for F344 rats. There is little specific literature on the effect of specific fats on food intake; however, it is clear that not all fats have the same effect on food intake. The effect of fatty acid chain length is one of the areas investigated. Both healthy and diabetic rats fed median chain triglyceride (MCT) oil preload consumed less food than those fed long chain triglyceride oil (LCT) [148,149]. Furthermore, diabetic rats fed MCT preload rapidly suppressed food intake during the first 2 hours whereas food

reduction after LCT preload was observed from 2-4 hours [148].In addition, rats fed the MCT-containing diet consumed significantly less food than LCT-fed rats[150].Friedman et al. proposed that different lipid delivery rates to the liver directly affected satiety [148]. MCT is more rapidly oxidized by the liver because it does not require acylcarnitine transferase to cross the mitochondrial membrane, which is a rate limiting step for LCT. Moreover, this process also enhances hepatic fatty acid oxidation [151]. A lower total fatty acid oxidation in the liver inhibits the mitochondrial transport of fatty acids, which has been shown to stimulate food intake in rats, suggesting that hepatic fatty acid oxidation is associated with satiety [152]. Therefore, the time between fat digestion and the next meal is an important factor in considering the effect of fat on satiety. The corn oil used in our study is a LCT, which explains the delayed response in food intake. Since both rat models consumed less total energy during the day, SD and F344 rats might have different rates of fatty acid oxidation processes in response to fat, which could have led to a different extent of energy compensation during the first 2 hours.

Consistent with previous studies [16,58], our data showed a slight increase in insulin levels and a significantly higher PYY level after oil preload [10]. While ghrelin was also significantly reduced by oil preload, this preload was associated with the highest active GLP-1 secretion although the effect was not significant. The similar hormonal responses between the two rat models were associated with different energy compensation (0-2 hour) but both rat models consumed less daily total food intake after the oil preload. Other studies have shown that rats fed a high polyunsaturated fat diet had higher POMC mRNA expression in the ARC compared to rats fed saturated fats and this promoted slower weight gain and lower food intake suggesting a direct involvement of fat in

neuropeptide regulation[153]. Collectively, the delayed satiety response associated with oil consumption may be mediated by its ability to stimulate insulin, PYY and GLP-1, as well as the direct involvement with neuropeptides. The lack of acute food suppression by reduced ghrelin levels indicates that the relationship between hormone levels and satiety is complex, suggesting that, if hormones play a role in food intake, the role is not a function simply of the circulating levels.

Macronutrient preload effects: Glucose

Similar to the case of oil preload, the effect of glucose differed between rat models: F344 rats showed energy compensation (150%) whereas SD rats did not (25%) for the first 2 hours. Glucose is mainly absorbed via two processes in the gut: sodium-dependent glucose transporter-1 (SGLT-1) and glucose transporter-2 (GLUT-2). In fasted rats, corticosterone induces protein catabolism that releases gluconeogenic amino acids, and stimulates the uptake of glucose by the small intestine. In rats, GLUT-2 is down-regulated in the fasted state whereas SGLT-1 (which has a high affinity for glucose and is associated with high levels of corticosterone), is up-regulated to promote glucose absorption even at low luminal concentrations. Therefore, glucose absorption is actively induced by the increased action of SGLT-1[154]. In this study, the lack of energy compensation observed in SD rats may be due to an insufficient amount of glucose in the preload to have an effect on food intake. However, there is evidence indicating that only carbohydrates with an affinity to glucose transporters have the ability to suppress food intake in rodents regardless of metabolic status, suggesting that glucose transporters play an important role in food intake regulation [155]. In addition to the potential differences in

apparent glucose metabolism between the two rat models, the basal level of corticosterone in F344 rats was 2 to 3 fold higher than in SD rats, which may have been associated with greater up-regulation of SGLT-1. A higher sensitivity to low glucose concentration may be one reason that energy compensation was observed in F344.

Both rat models had similar hormonal responses to the glucose preload. In agreement with the scientific literature, both rat models fed the glucose preload had significantly higher insulin levels after preload but there was no effect on PYY secretion [9,58]. However, differing from the scientific literature, active GLP-1 levels were unaffected in either rat model. In addition, the levels of ghrelin were suppressed only in SD rats even though it is known to decrease in response to food intake [16]. Interestingly, the glucose preload decreased food intake during the first two hours only in F344 rats. This suggests that the food-associated inhibition of F344 rats after glucose preload is only due to changes in insulin levels since changes in ghrelin did not contribute to changes in food intake in SD rats. It is thought that glucose suppression of food intake during the first 2 hours in F344 rats did not translate into a prolonged inhibition of energy intake throughout the day because glucose is rapidly absorbed and metabolized/stored after stimulating insulin secretion[155].

Due to the limited number of rats, I reasoned that since glucose had the largest effect on insulin levels, only the effect of water (as control) and glucose on mRNA expression would be measured. Glucose injections mimicking a similar elevation of glucose as seen after a carbohydrate enriched meal effectively suppressed NPY and AgRP mRNA in the ARC up to 60 minutes post injection [156]. However, this was not observed in rats fed a high carbohydrate diet where there were no obvious changes in

NPY and AgRP mRNA expression [157]. In this study, neuropeptide mRNA expression was not affected by glucose preload in either F344 or SD rats. Perhaps under normal feeding circumstances, a much higher concentration of glucose is needed to induce noticeable changes in neuropeptide mRNA expression. Even though injection of glucose indicates that a link exists between glucose and neuropeptides, this method is more direct and bypasses the gut altogether. It is common for studies to use a more direct method of delivery to ensure a large response. Studies often deliver nutrient via direct infusion to the duodenum, which would provide a much higher concentration of nutrients in a short time, to better identify the link between gut hormone secretions and nutrient exposure. Therefore, glucose related increases in active GLP-1, may have been observed because of excessive levels of nutrient in the GI tract. Because the infusion paradigm does not mimic a normal physiological process, where glucose is more slowly released into the upper GI tract because of the effect of gastric emptying and the rate of glucose absorption, glucose may not reach the distal part of the GI tract in high concentrations [51]. Thus, different nutrient delivery methods can contribute to vastly different outcomes. This conclusion is supported by a study that found glucose gavage did not elicit a change in GLP-1 secretion, but a combination of glucose and oil significantly increased GLP-1 release (the effect of oil alone was not reported)[17]. Delayed glucose absorption brought about through the addition of oil to the GI tract may have played a role in aiding glucose induced hormone changes by exposing the lower GI tract to higher glucose concentrations. This study also found that GLP-1 concentrations in the tail vein were significantly lower than those measured in the inferior vena cava or portal vein [17]. My study took serum samples from the tail vein, which may have hindered the ability to detect GLP-1 measurements.

Although active GLP-1 was released in a dose dependent manner upon glucose infusion, a duodenal glucose infusion at levels similar to those used in my study took up to one hour to reach a GLP-1 level significantly higher than control[48]. Serum samples from this study were taken at 30 minutes. This may have not been long enough for the rats to attain a higher level of GLP-1 production. An interesting study investigated the sensitivity of GLP-1 release in response to different types of carbohydrates given by ileal infusion. Glucose induced significantly higher levels of GLP-1 after 10 minutes, but these returned to baseline at 30 minutes. At the same time, fructose and galactose increased GLP-1 more gradually and levels remained elevated at 30 minutes [59]. Even though it is difficult to compare studies with different protocols, this study showed that response time can differ among different types of carbohydrates. The correct time to take the measurement becomes a crucial factor in obtaining relevant results. Therefore, the lack of an expected glucose effect on active GLP-1 secretion my study may be related to tail vein blood sampling taken at 30 minutes post gavage and/or an insufficient (but physiological) magnitude of glucose delivery to the lower GI tract.

Significant correlations between acute food intake and hormones

In F344 rats, insulin secretion was positively correlated with food intake after rats were fed a water preload (control). This is in accordance with the literature which suggests that insulin release is directly associated with food intake with its main function being to attenuate the postprandial rise of glucose [8]. Even though exogenous ghrelin administration has been shown to increase food intake dose dependently [26,29,30]and ghrelin is suppressed by food intake [24], a negative relationship between ghrelin and food

intake was not observed in either rat model in this present study. One possible reason is that higher food intake might be required to observe a noticeable change in ghrelin. Studies have shown that injection of ghrelin at high levels has physiological effects [158]. Therefore, even though a relationship exists between ghrelin and insulin, the association may not be a strong one under normal circumstances. In my study, I saw that ghrelin and insulin levels were positively correlated after water and glucose preloads. This suggests that insulin and ghrelin may respond to signals other than food intake alone. The linear correlation between active GLP-1 and insulin after water preload is in keeping with GLP-1 acting as an incretin, which has insulinotropic activity that enhances insulin secretion during food consumption [39].

Correlations between active GLP-1 and total PYY after water, glucose and protein preload were the only associations observed in both rat models. GLP-1 and PYY are both anorexigenic peptides secreted from the same endothelial cells in similar sections of the GI tract. It is not surprisingly to find that they are secreted in the same manner in response to food intake. Total PYY was significantly elevated after rats were fed oil preload whereas active GLP-1 showed a trend towards increasing but this was not significant. Therefore, a correlation was not observed between the two hormones after oil preload. This implies that, while fat has a tendency to trigger an increase both gut hormones, PYY secretion is more sensitive to fat. It is important to note that neither active GLP-1 nor total PYY correlated during the first two hours of food intake. This further confirms that protein inhibited food consumption during the first 2 hours of feeding through factors other than active GLP-1 or total PYY.

The lack of strong correlations between other hormones may arise because each nutrient elicits a different hormonal response and the relative strength of this response may differ among rats. As a result, the interactions between macronutrient and hormone secretions may not always give a linear correlation.

The effect of fermentation on long term food intake

The focus of my study was to assess physiological responses to gastrointestinal exposure, specifically through behaviour (food intake) and hormonal control of this behaviour. However, the current study omitted any investigation of a possible underlying mechanism. One important factor in fermentation is the microbial community in the cecum of rats. My data showed that both rat models fed the FOS supplemented diet consumed significantly less daily total food and energy than rats fed the WB diet. Both rat strains fed the FOS diet also consistently consumed less food than rats fed the CTR diet. In contrast, the WB diet did not have the same effect as FOS in food intake; it actually promoted food intake for both rat models albeit that the neither FOS nor WB diet led to significant reduction in food intake or change in weight gain. Nevertheless, the FOS fed-SD rats consistently gained less weight and body fat compared to the other diets. Studies investigating the role of community composition on physiological responses include oral supplementation with Bifidobacteria and Lactobacillus, which attenuated hepatic fat accumulation in rats [159]. Studies such as this one, suggest that specific gut associated bacteria may play a role in nutrient metabolism and potentially in hormone release. The link with fermentation is obvious since it is well known that DF can modulate the gut microbial community [160-163]. In particular, FOS fed rats showed

significantly higher Lactoacilli counts[162] and increased butyrate production [161]. Butyrate is one of the main SCFA produced in the colon during DF fermentation. Modulation of SCFA production, the main energy source for colonocytes, has also been shown to control gene expression. For example, Chen et al. showed that rats fed 5% FOS and 10% WB diets (same amount of fermentation as my study) had very different changes in gene expression profiles in terms of both degree (the 5% FOS diet was associated with more changes in gene expression than the 10% WB diet) and specific gene responses, including genes involved in the nutrient and SCFA transport in the proximal colon [164]. This suggests that hormone production could also be influenced by changes in bacterial composition leading to alterations in the SCFA profile.

Direct effects of FOS (and consequently the rate and extent of fermentation) on food consumption have also been noted before with mixed results in rats. For example, Ohta et al. showed that SD rats fed the 5% FOS diet consumed significantly less food than controls [165] whereas Lobo et al. showed that a 5% FOS diet did not affect food intake in Wistar rats [165,166], suggesting that the effect of fermentation may be strain dependent. The observation that rats fed a WB diet consumed more food has also been previously reported [167,168]. However, this is usually associated with very high DF intakes (20% WB diet) and these diets were not controlled for energy intake. My study compared the effect of DF at identical levels of fermentation and calorie content. Taken together, both FOS fed F344 and SD rats consumed significantly less food intake than WB fed rats, but only SD rats showed a reduction in weight gain. This suggests that strain differences in the utilization of SCFAs or in the metabolic influence of SCFA on energy deposition may have contributed to altered weight gain since this was the only factor that differed. The

effect of fermentation of food intake is rate dependent as WB fed rats consistently had a higher food intake.

The effect of fermentation on hormonal responses

WB did not have any influence on any hormones measured in this study. The only effect of FOS on hormonal change was a significant increase in circulating total PYY in F344 rats. I did not observe a fermentation effect on active GLP-1 production. Previous studies have shown that a 10% FOS diet significantly increased active GLP-1[116,117]. On the other hand, diets containing 9% inulin [169] or 6% GOS [47] did not affect GLP-1 secretions [169]. These results suggest that rapidly fermentable material may be effective in promoting basal GLP-1but only when given in very high amounts. It is possible that the 5% FOS diet used in my study might not have provided sufficient fermentation to elicit a significant increase in active GLP-1.While GLP-1 did not respond, rats fed both the 6% GOS diet and 10% FOS showed a significant increase in the basal level of PYY [47,170]. A diet containing 30% resistant starch also led to increased PYY and GLP-1 levels [119]. Although evidence regarding the effect of WB is lacking, DF is clearly linked to gut hormone production. Since I showed that hormone production in F344 rats fed the WB diet was not affected, I conclude that in addition to the total amount of fermentation, the rate of fermentation also seems to be an important factor. The increase in basal total PYY levels in FOS-fed F344 rats, at least in part, can be attributed to the decreased average daily energy intake compared to the CTR or WB diets over the long term.

Relationship between mRNA and peptide productions

Ghrelin mRNA expression in the stomach was not reduced by the glucose preload in either rat model. This is opposite to what is generally accepted as the effect of glucose on ghrelin. As an indicator of hunger, ghrelin mRNA expression is the highest during the fasted state and reduces with exposure to food [158]. In the Lee study, the rats were fasted for 72 hours, and this may explain the differences between their study and ours. Despite the fact that ghrelin mRNA expression was not changed, ghrelin peptide levels were significantly reduced but only in SD rats. Thus, unaltered ghrelin mRNA expression is consistent with the lack of an effect on ghrelin peptide levels in F344 rats but not in SD rats. The lack of a correlation between mRNA and hormone release was expected, considering other results showing a correlation between gene expression on the mRNA level and protein level in 71 human genes varied greatly for genes in different biological categories [171]. Genes defined within each category was not specified, nonetheless, genes involved in cellular components of the cell structures had the highest mRNA-protein correlation. On the other hand, genes involves in regulation of biological processes had the lowest correlations [171]. Therefore, while mRNA expression can be a useful indicator, it does not predict protein expression perfectly.

The only significant changes in neuropeptide mRNA levels occurred in the ARC of FOS fed F344 rats where AgRP and NPY mRNA expression were much higher than those in WB fed rats. AgRP and NPY are known orexigenic peptides [87] and intracerebroventricular injection of AgRP prompted a significant increase in food intake in SD rats [32]. This is in contrast to my results, where FOS-fed F344 rats had significantly lower short and long term energy intake compared to WB. This suggests either that the

differences in mRNA expression are strain-specific and not related to altered food intake or that the mRNA levels do not translate into high enough levels of peptides to exert the orexigenic effect in F344 rats.

The cecum was the only tissue in the lower GI tract that was greatly impacted by fermentation and/or glucose preload in both rat models as assessed by changes in PYY and PGC mRNA levels. The fact that PYY and GLP-1 peptide secretions are dependent on the type of nutrient is widely accepted [70]. However, evidence is lacking on the regulatory effect of nutrients on mRNA levels. In the present study, relative mRNA levels of PYY and PGC were significantly elevated in the cecum after FOS feeding in both F344 and SD rats. Nutrient gavage showed that glucose tended to increase PYY and PGC mRNA expression (it was only close to statistical significance for F344 rats). In addition, glucose and FOS appeared to enhance PYY and PGC mRNA levels additively. The results are in keeping with studies showing that fermentation increases PGY and PYY mRNA levels [45,47]. For example, a study with resistant starch-fed SD rats showed an up-regulation of PYY and PGC mRNA levels in the cecum and colon, but not in the small intestine [45]. FOS diets fed Wistar rats showed significantly increased PGC mRNA expression in the cecum and proximal colon [116,117,170,172,173]. FOS is rapidly and completely fermented in the cecum of rats and the consistent mRNA increase in the cecum of different rat models supports the idea that fermentation plays a role in modulating PYY and GLP-1 at least at the mRNA level. Note that the study that found an increased PGC mRNA expression in the proximal colon had fed the rats diets containing 10% FOS. As was discussed earlier, the much lower FOS content in this current study may be one reason why changes in mRNA levels were not observed. A link between fermentation

and hormone mRNA levels implies a direct effect of short chain fatty acids on gene expression. SCFAs are the major end products of fermentation and represent the principal source of energy for colonocytes [174]. Even though the WB and FOS diets were designed to have the same total amount of fermentable material, FOS fermentation produces more butyric acid than does WB and butyric acid is the main SCFA source in the lower GI tract and the principal energy source for colonocytes [122]. In addition, rapid fermentation of FOS may produce a locally high (and possibly shorter-lived) SCFA concentration in the cecum and proximal colon. This might explain the lack of mRNA changes in rats fed WB diets. [122]

One study suggested that diets containing 10% FOS have specific effects in promoting PGC mRNA expression because there was a lack of PYY mRNA expression change along the lower GI tract [170]. Their main interest was in Bifidobacterial effects on the GI tract, bacteria which are not abundant in the rats from this experiment, even after feeding 5% FOS (about 4%, data not shown).

In my study, I observed increased PGC and PYY mRNA levels in both rat models fed 5% FOS. It should be noted that the significantly elevated mRNA expression only correlated with significantly higher PYY levels in F344 rats. Furthermore, neither total PYY nor active GLP-1 secretions were enhanced by glucose as seen when examining the mRNA expression profiles. Thus, it appears that F344 and SD rats do not share the same mechanism of protein modification, at least in terms of circulating PYY and GLP-1 in response to fermentation. This suggests that the rate of fermentation has a strong impact on PYY and GLP-1 at both mRNA and protein levels, but this does not necessary translate into hormone production. The reasons might be that: 1) mRNA does not always

translate into protein, and 2) active GLP-1 is measured rather than total GLP-1 and more than 50% of GLP-1 can be inactivated by DDPIV when released from intestinal endocrine cells [39].A more detailed investigation of the relationship between mRNA and protein levels of these hormones needs to be done before relying on mRNA transcript levels as the sole indicator of hormonal production.

Distribution of PYY and GLP-1 in the lower GI tract

While it is generally accepted that PYY and GLP-1 are mostly produced in the lower GI tract [70], precise sites responsible for PYY and GLP-1 production and mRNA expression pattern are still unclear. My study found the highest PGC mRNA expression levels per 1μg of RNA in the lower GI tract. At the same time, the expression had a tendency to decrease along the GI tract with the distal colon being the lowest mRNA production site for both F344 and SD rats. The same trend was observed for PYY mRNA expression in SD rats but not in F344 rats, where the ileum and colon had higher PYY levels than cecum. Examination of the expression profile in SD rats previously showed that PYY mRNA expression was lowest in duodenum and increased distally with colon as the main production site. PGC mRNA expression was the highest in jejunum and second highest in the colon [45].

Even though cecum did not appear to be the dominant site of those mRNA productions, a study by Onaga et al. showed that the PYY content per gram of tissue increased towards the distal end of GI tract, and the PYY content in the cecum and colon were much higher than the ileum in both humans and rats [68]. The main GLP-1 production site appears to be the proximal colon [116,170]. In my study, effects on PYY or GLP-1 were

only observed in rats fed the FOS diet. The cecum is significantly enlarged by the FOS diet and appears to be an important site of gut hormone production, at least when fermentation is involved. The cecum's importance in hormone production is further supported by the results from the F344 rats: cecum of the FOS fed rats produced similar level of PYY in the cecum as primary production site while it is the lowest PYY production site for the CTR or WB fed group. The enlarged cecum in rats fed FOS has been noted by other studies [116,172], and a larger physical size of the tissue would have more endocrine cells available to produce a higher level of the hormones overall. Because mRNA expression was up-regulated in the cecum, the cecum may be the site responsible for the significant increase in circulating PYY levels noted in animals fed FOS. Two possibilities may explain why the mRNA expression did not reflect the actual hormone levels are: 1) While ileum expressed the highest mRNA levels, it retains very little of the hormones produced in the tissue because it rapidly distributes them into tissues or circulations; and 2) The mRNA was not completely translated into proteins in ileum while cecum or colon may be more efficient in protein translation.

Linking gut hormones, macronutrients, fermentation, and food intake together

Although FOS fed F344 rats and F344 rats given an oil preload showed increased total PYY levels, the FOS and oil preload effects were not additive for PYY release. The same effects were observed for food intake reduction. Food intake was reduced by both FOS diet and oil preload but the interaction did not reduce food intake additively. Because the effect of oil preload on food intake was not observed from 0-2 hours, it is difficult to interpret whether the higher level of PYY associated energy reduction was

primary due to the FOS diet or the oil preload. However, it is unlikely that the reduced food intake solely relies on either FOS or oil induced PYY production since the inhibition of food intake was not observed in FOS fed rats after glucose or protein preload. Likewise, WB fed rats after oil preload also did not show reduced food intake. Since there was no additive interaction effect, it is possible that neither of the stimulants reached the maximal release of PYY. This argues for a threshold effect of PYY and not a linear dose-response relationship. Rats fed a high fat diet containing an additional 10% FOS significantly reduced energy consumption and weight gain when compared to a high fat diet alone [116]. This suggests that rather than acting alone, a combination of FOS and fat may be the key to successful food inhibition.

Energy intake and PYY did not respond in the same fashion for SD rats. The increase in total PYY levels brought about by oil preload did not translate into a reduction in energy intake. In addition, although the average daily energy intake was reduced in SD rats fed FOS diets; this was not associated with a higher basal PYY level compared to other diets. As a result, food intake for SD rats appears to be influenced by factors other than PYY release only. Unlike F344 rats, the only hormonal interactions observed in SD rats were between PYY and GLP-1. Other evidence has shown that food intake is closely associated with the hormones in this present study. If those hormonal responses to nutrient are not as sensitive or responsive in SD compared to F344 rats, this may explain the lack of an association in SD rats.

Rats fed a 6% GOS diet show significant increases in total PYY release, but not active GLP-1[47]. In these rats, after the fall of PYY following a peak at 60 minutes post meal, PYY gradually increased again at 90 minutes and remained elevated and

significantly higher than control at 240 minutes post meal [47]. This study suggests that FOS fed F344 rats (of this current study) with higher basal total PYY levels may have had a higher, prolonged steady state of circulating PYY. These factors altogether were able inhibit energy intake longer. Another plausible reason that SD fed oil preload led to significantly higher circulating total PYY but did not translate into food inhibition in my study is that the FOS diet did not stimulate higher total PYY secretions. Perhaps SD rats achieved energy compensation by increasing food consumption after the rise of total PYY stimulated by the oil preload had returned to baseline. This further confirms that although FOS diet and oil preload do not inhibit energy intake additively, the interaction may have a significant role in prolonged energy inhibition.

Active GLP-1 did not seem to play a role in mediating short term or long term food intake in this study because its levels did not change under any condition. Nevertheless, both F344 and SD rats fed oil preload were associated with the highest active GLP-1 secretions although this effect was not significant.GLP-1may function synergistically with PYY and could be a factor in modulating food inhibition. Exogenous administration of PYY and active GLP-1, at doses that have no effect on food intake individually, showed significantly reduced food intake additively in both human and rats [53,81]. Therefore, the contribution of active GLP-1 to food inhibition cannot be ruled out based on the lack of significant increase in the hormone in the present study.

Nutrient composition is important in modulating the postprandial rise of circulating hormones. Different studies using different control diets, different nutrient loads and different delivery methods have shown mixed results in the same animal model due to the nutrient and localized action of PYY and GLP-1 secretions. Moreover, strain

differences also contribute to different responses under the same experimental conditions as observed in my study. This has led to difficulties in drawing a consensus conclusion on gut hormone release in response to food intake. Although hormonal responses could be species- or even strain-dependent, the different experimental designs also mask the possible underlining universal mechanism of gut hormones. Thus, in the case of F344 rats, oil suppressed active ghrelin equally to protein but oil did not reduce food intake the way that protein did. Similarly in SD rats, both glucose and oil significantly suppressed ghrelin but this did not translate into subsequent food inhibition. Furthermore, hormonal responses after glucose and protein preloads were very similar in SD rats, but only protein had an effect on short term satiety. Taken together, my data shows that appetite control is a complex system that involves many other hormonal interactions. Ghrelin suppression does not always translate into food intake inhibition. Likewise, higher PYY or GLP-1 levels do not necessarily reduce food intake linearly. More hormones involved in food intake should be considered in mapping the potential mechanism of food inhibition. Nevertheless, the present study provides links and possible interactions between food intake and hormonal changes in response to DF and macronutrients under normal physiological conditions. The results suggest that satiety-associated hormones have important roles in food regulation, but no single hormone or pathway is a clear and inclusive indicator of food intake in response to hunger or appetite.

Corticosterone and blood glucose

Even though glucose preload did not result in higher blood glucose levels compared to controls, it still induced the highest insulin release as expected. Because the

glucose response was so low, the same glucose concentration (and solution) was given to 2 adult Wistar rats twice on separate days in the same manner to ensure that errors in preparation of the glucose solution itself was not the reason for failure in blood glucose response. The Wistar rats showed significantly higher blood glucose after glucose preload (data not shown). At first, it was suspected that, in addition to the action of insulin, the rats had other strain dependent underlying mechanisms to quickly metabolize the glucose. However, this phenomenon was not observed for necropsy serum samples – in these samples the blood glucose level was indeed higher after glucose preload. Rats fed the FOS diet had significantly suppressed blood glucose but only in tail vein samples from SD (day of preload) and in necropsy samples (core samples) of F344 rats. Although the effect of FOS indicates that fermentation can play a role in mediating blood glucose, the discrepancy in blood glucose levels between samples obtained at different time points from the same rats was unexpected. Blood glucose has been shown to increase in rats under physical or chemical stress [175]. Thus, one reason for the lack of a consistent glucose effect may have been related to the stress induced during the tail vein blood sampling – this could have been high enough to mask the rise of blood glucose by glucose preload. Corticosterone is a hormone released in response to factors such as stress and is used as the stress indicator in rodents [126]. However, corticosterone levels were higher during trunk blood collection at necropsy as compared to tail vein sampling and this effect achieved significance in F344 rats. The effect is opposite to predictions and the reasons remain unclear. An interesting finding is that rats fed WB had noticeably lower corticosterone levels than FOS or CTR diets. Whether WB has a potential role in mediating stress remains to be further investigated.

Human implications

Many human studies have been conducted to identify the link among gut hormones, choice of diet and short term satiety. These studies face similar challenges to the animal studies in term of interpreting the results where the direct links are clear at a superaphysiological level that is not easily achieved in everyday life.

If diet modification is to be used as a weight management tool, it is important to understand the effect of each macronutrient. Foods with a high glycaemic index (GI) have been associated with an increased risk of obesity since glucose elicits a series of hormonal events that challenges glucose homeostasis over the long term. These effects can include a high level of blood glucose associated with a large glucose load. This high postprandial rise in glucose often starts to decline within 1 hour to a level below a fasting state. This "crash" is thought to induce hunger and promote higher daily food consumption [176]. Confirmatory data from rats fed a high GI diet showed a significantly higher fat mass and a higher blood glucose response after an oral glucose intake [177]. My study found that while protein elicited increased insulin secretion, it also lowered blood glucose in both rat models. This is consistent with the literature which indicates that proteins are generally effective in stimulating insulin release and can be just as effective an insulin stimulant as glucose without increasing blood glucose concentrations [11].

Protein is generally accepted as the most satiating macronutrient and is promoted to suppress appetite. However, there is limited evidence from human studies that the acute effect of satiety translates into long term benefits. Thus, published studies have shown mixed results on the role of macronutrients on weight loss. For example, an intermediate study of 12 weeks showed that a high *ad libitum* protein diet (50%

CHO/30% protein/20% fat) without energy restriction successfully reduced weight in healthy men. It was proposed that the effect of increased protein over time lowered caloric intake by as much as 1850KJ per day relative to baseline without the need to restrict energy intake [8]. However, in a similar cohort study of 12 weeks with overweight subjects consuming an isocaloric diet of 5600 KJ, there was no difference in weight loss between a high carbohydrate (64% CHO/17% protein/20% fat) or high protein containing diet (46% CHO/34% protein/20% fat). Moreover, high protein diet promoted health benefit such as significantly reduced level of triglycerides independent of weight loss [178].

Fat is a potent stimulant of PYY and GLP-1 secretion and a weeklong diet composed of high fat and low carbohydrate (up to 15% total calories per day from CHO) significantly increased postprandial PYY compared to the high carbohydrate and low fat diet (up to 20% total calories per day from fats) [179]. Because the food intake data were not recorded, it is hard to interpret the effect of increased gut hormone productions. However, the high fat effect is not always seen. For example, a high fat diet does not always translate into weight loss over the long term. The effect of a low carbohydrate, high protein and high fat diet (Atkin's diet) and a high carbohydrate and low fat diet (control) on weight changes of obese men was investigated over 1 year. Total energy consumption was not restricted but the Atkin's diet included a restriction on carbohydrate consumption. The control diet was approximately 60% CHO, 25% fat and 15% protein. The group on the Atkin's diet lost significantly more weight up to 6 months, but the weight changes were not different between the two groups at 12 months [180]. Another 12 month cohort study did a similar comparison using isocaloric diets of 4% CHO, 35% protein and 61% fat versus 46% CHO, 24% protein and 30% fat. There was also no significant difference

in weight loss between the two groups [181]suggesting that a long term adherence to a specific diet may be difficult [180]. One study, investigating the effect of 4 different macronutrient compositions on weight loss over two years with more than 800 participants, showed that while all 4 diet groups lost weight, there was no difference in weight loss or waist circumference reduction among the groups at 6 months or at the end of 2 years [182]. All those results suggest that weight loss over time can be achieved more by simplifying the diet plans or restricting energy intake regardless of macronutrient composition.

High fat diets equal in palatability with control diets have been shown to increase energy intake in rats [134] and humans [183]. Furthermore, dietary fat is less satiating and higher in calories per gram and increased fat content in the diet increased energy consumption dose dependently [183]. In humans, higher fat content increases the risk of obesity that usually leads to higher fat mass and weight gain [184]. This is problematic because obese men have significantly lower basal levels of ghrelin, and a blunted hormone response to food as shown for ghrelin [33], PYY [185] and GLP-1 [50] when compared to healthy men, albeit that fat is a potent PYY and GLP-1 stimulant. Obese men also have a much lower postprandial insulin response [50]. The higher hormone levels over time may lead to reduced efficacy and desensitization of the satiety signals. The impaired ability to properly respond to physiological cues may result in delayed satiety signals and lead to an increased food intake in order to achieve the satiated state brought about by control levels of gut hormones. This may promote an earlier onset of the next meal and, ultimately, a higher prevalence of obesity. Although overconsumption of dietary fat is linked to obesity, it is important to recognize that it has a significant role in suppressing

food intake other than its effect on PYY and GLP-1 secretions. Fat digestion is essentially a prerequisite for PYY secretion and proper ghrelin suppression. Duodenal infusion of fat with lipase inhibitor showed that inhibition of fat digestion lead to elevated ghrelin levels and a fixed level of total PYY at baseline in healthy men [186].

Strengths and weaknesses of the current study

The energy intake of both strains was very similar within diet groups, but clearly dropped around the mid-point of the study and then remained constant at the new level. The mid-point was the start of the preload experiments, but it was not responsible for the drop in energy consumption. The same trend was also observed in 10 CTR diet fed F344 rats that were kept for a behavioural reference (data not shown). These rats were not fed gavage preloads. A possible explanation for the declined energy consumption is that rats required less energy once they had passed the rapid growing phase and gradually reached maturity, indicating that this was a suitable time point at which to introduce the short term satiety experiment where the bias of rapid eating and weight gain during the growing phase on food intake can be excluded. Although eating pattern varies among rats, each rat followed its own eating pattern consistently (data not shown, but was analyzed based on the 3 occasions that rats were gavage fed with water). Rats were chosen as an animal model because of the similar anatomical characteristics and physiological responses to humans. Furthermore, there is a large database of rat data, which is useful for comparative purposes [130]. For those reasons, rats are a very suitable model to use for investigating factors that affect food intake given that every rat acts as its own control. The one disadvantage is that rats consumed relatively little weight of food. The statistical

significance of an effect can be altered by merely a 0.5 gram difference in intake, which increased potential errors in food intake measurements. Thus, multiple trials of the preload experiment should be repeated in order to obtain a more confident result.

This study also used two different strains of rats, both inbred and outbred. Inbred F344 rats are produced by brother X sister mating, thus the strain is virtually isogenic. The low genetic variation among rats is an attractive property for studying gene expression responses. The greater consistency in physiological responses leads to a higher statistical power and enables detection of a given biological effect with fewer animals [130]. However, since humans are not inbred (at least not in the greater Ottawa area☺) and are much more genetically variable, an isogenic strain is not the best representation of the human population and can lead to false conclusions. Since stress, drugs, and chemicals induce different physiological responses in SD and F344 rats [130], it is not surprising that different outcomes were observed in the present study. However, differences and similarities between these two rat strains probably give a more realistic picture of hormonal effects and variability that might be extrapolated to the human condition. Moreover, rats are excellent animal models for studying the effect of diet on energy consumption because the pattern of food consumption is constant. This could be due to the fact that they are fixed on specific diets and food consumption is driven strictly by hunger. In humans, food consumption can also be driven by factors such as opportunity, social events, physiological differences, upbringing, stress, external cues and organoleptic qualities of food. This means that caution should be taken when interpreting the results from published rat studies.

Summary of findings

Both rat models fed the WB diet appeared to exert an opposite effect on food intake than FOS diet and seemed ineffective in modulating hormone levels. FOS fed F344 had higher basal PYY levels while none of the hormones studied were affected by WB diet in either rat model. In addition, although there was no significant difference in weight among the groups, rats fed WB diet consumed higher total daily energy over the long term. This suggests that WB did not have beneficial roles as compared to the CTR diet used in this study.

Acute food inhibition (0-2 h) was the strongest following the protein preload in both rat models. Both F344 and SD rats fed the FOS diet had lower long term food intake and higher PYY and PGC mRNA expression in the cecum than CTR and WB fed rats. The F344 rats were more sensitive to the FOS diet in terms of circulating PYY stimulation but body weight gain was unaffected. In contrast, FOS fed SD rats did not have a higher basal PYY level but had reduced body weight. Both F344 rats and SD rats fed the oil preload showed the greatest total PYY and active GLP-1 secretion but food intake inhibition was only observed for F344 rats. Altogether, these data suggest that the mechanisms that mediate the relationship between the amounts of nutrient, gut hormone releases, and food intake may not be universal.

Conclusions

The scientific literature has not provided strong evidence for a link between fermentation and macronutrient composition that helps protect against weight gain over the long term. This implies that a healthy lifestyle with controlled weight gain is

dependent on a combination of factors. In my study, it appeared that an interaction between fibre and nutrient was important in mediating hormone secretion, suggesting that it may be a useful tool for weight control. Rats fed both WB and FOS diets had lowered blood glucose. The action of active GLP-1 is to enhance endogenous insulin secretion to lower blood glucose. These are favourable traits for treating diabetic patients. Prescribed active GLP-1 agonists have been successful in treating diabetic patients as a weight control tool [70]. It would be of great benefit if the stimulation of endogenous active GLP-1 by nutrients in the gut could be achieved naturally to a high enough level to act as a therapeutic agent. Whether the effect of the gut hormones can be achieved by increased basal levels or by the additional stimulation past the basal value requires further investigation.

It is difficult to interpret the relationship between diet compositions, weight control and hormone levels in human cohort studies due to insufficient data from the same study. Studies do not usually conduct multiple interventions and studies often investigated changes in the levels of one or two hormone secretions after a week of diet intervention without measuring its effect of food intake or vice versa. Long term cohort studies also tend to use self-reported satiety ratings rather than measures of gut hormones. Most studies that investigate the relationship between food intake and gut hormone release utilize over exposure of nutrient or hormone via injection or infusion. Although a clear correlation can be made, the physiological changes related to over exposure do not represent the normal physiological state.

My study, using normal nutrient levels confirmed some of the literature findings and added new insights. The confirmed findings include: 1) protein is a potent short term

satiety stimulant. 2) PYY, GLP-1, ghrelin and insulin responses are nutrient dependent. 3) Oil is a potent stimulant for PYY and GLP-1 secretions. The new insights are 1) Responses can be strain dependent since the outcomes were not the same for F344 and SD rats. Thus, the underlying mechanism may not be universal. 2) The rate of fermentation plays an important role in hormone release. Rapidly and completely fermented material, such as FOS, had an impact on PYY and PGC mRNA levels and PYY release. 3) Increased basal levels of PYY did not affect short term satiety. 4) There appears to be an interesting interaction between FOS and oil to prolong inhibition of food intake. 5) Total PYY and active GLP-1 secretions in response to glucose and protein are strongly and positively correlated. 6) Both WB and FOS are candidates for lowering blood glucose, but the effect of fermentation on food intake and hormonal response is rate dependent. 7) In addition to mRNA expression measurements, protein measurements should also be done wherever possible.

It is clear that different rat strains can have different physiological responses. Therefore, results from rat studies should be interpreted with caution when comparing to humans. It is also important to note that humans are likely to have a wider range of responses due to the large differences between individuals in lifestyle, diet, ethnicity and race. No simple relationship among different hormone levels and satiety or long-term food intake was observed, suggesting that, if hormones play a role in food intake, the role is subtle and not a function simply of the circulating levels. In addition, it appears that different macronutrients elicit different hormonal responses suggesting that no single pathway for controlling satiety exists.

Future directions

In the future, it would be advantageous to examine the effect of food inhibition by macronutrients if rats were allowed enough time for gut hormones to peak before onset of the dark cycle. To do so, time required for gut hormones to reach the peak values in short intervals in response to food consumption should be determined. It is also important to know if different macronutrients require different amounts of time to elicit hormonal responses. Whether dosage of different macronutrient preloads translates into dose dependent or prolonged hormone secretions that affect food intake should also be determined. All these factors can further confirm whether there is a threshold effect of hormones on food intake. Because protein and oil elicited bigger responses than glucose, mRNA expression of the neuropeptides and hormones after protein and oil preload should also be examined. To further test for interacting effects of FOS and oil on food intake, this procedure should be repeated over a longer period of time to investigate whether this interaction translates into long term reduction in food intake or body weight. The gut bacterial community should also be determined to explore the possible relationship between the results obtained so far and bacterial populations.

Appendix I

Energy compensation equation

All food intake (in grams) were first converted to energy density (kcal)

Energy compensation =

$$\frac{\text{Energy consumption (water)} - \text{Energy consumption (glucose)}}{\text{Energy density of preload (glucose)}} \times 100\%$$

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