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CHRONIC HIGH FRUCTOSE FEEDING INDUCES NEITHER A POSITIVE SHIFT IN ENERGY BALANCE NOR LEPTIN RESISTANCE IN MICE

by

ERIK J TILLMAN

Steven J. Swoap, Advisor

A thesis submitted in partial fulfillment
of the requirements for the
Degree of Bachelor of Arts with Honors
in Biology

WILLIAMS COLLEGE

Williamstown, Massachusetts

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ASTRACT

Chronic 60% fructose feeding decreases the sensitivity to leptin in rats as assessed by decreases in food intake and body weight after administration of exogenous leptin. We hypothesized that a 60% fructose diet would also lead to leptin resistance in mice, as assessed by 1) a lack of response to exogenous leptin, 2) elevated circulating leptin, and 3) a lack of entry into torpor upon fasting. In experiment 1, male Hsd:NSA (CF-1) mice were fed either a 60% fructose diet or an isocaloric fructose-free diet and food intake and body weight were monitored for 3 weeks. In experiment 2, male C57Bl/6 mice were fed either a 60% fructose diet or an isocaloric fructose-free diet and food intake and body weight were monitored for 14 weeks. After respective feeding periods, mice in both experiments were injected interperitoneally with leptin (5 mg/kg) and consequent changes in food intake and body weight were monitored. High fructose feeding did not attenuate the expected hypophagic response to exogenous leptin. These observations indicate the failure of a 60% fructose diet to induce leptin resistance in a 3 week feeding period in Hsd:NSA (CF-1) mice or over a 14 week feeding period in C57Bl/6 mice.

INTRODUCTION

Energy Balance and Satiety

Mammals have evolved tight regulatory mechanisms for maintaining energy balance, the difference between energy intake and energy expenditure. Energy intake is calories gained from feeding, and energy expenditure includes physical activity, adaptive thermogenesis, and resting metabolism. Physical activity encompasses voluntary movement, adaptive thermogenesis is the production of heat through the dissipation of energy in response to environmental factors, and basal metabolism includes all biochemical processes necessary for the sustenance of the life of the organism (Spiegelman and Flier 2001). A positive energy balance corresponds to a greater energy intake than expenditure, and chronically results in weight gain; a negative energy balance represents greater energy expenditure than intake, and corresponds to weight loss (Figure 1).

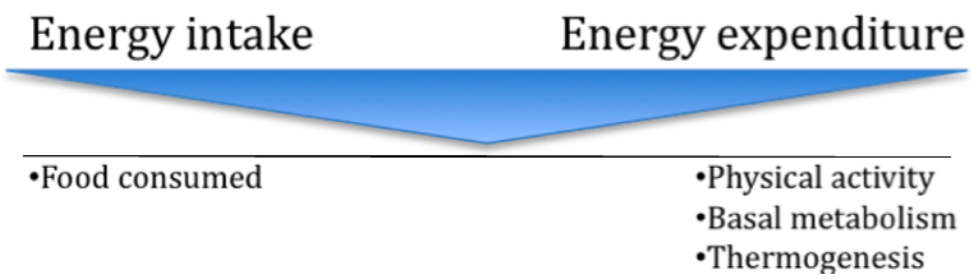

Figure 1- Schematic of energy balance. When energy intake chronically exceeds energy expenditure, obesity ensues. If expenditure chronically exceeds intake, starvation occurs.

The central nervous system (CNS) effects stable energy balance via three mechanisms: 1) behavioral effects, including altered feeding patterns and activity; 2) regulation of autonomic nervous system (ANS) control of thermogenesis and metabolic processes; and 3) neuroendocrine effects on appetite and satiety. The hypothalamus is the center of energy balance regulation, suggesting a feedback mechanism linked to peripheral fat stores whereby feeding behavior is altered by the CNS in response to energy imbalance (Kennedy 1953); Figure 2).

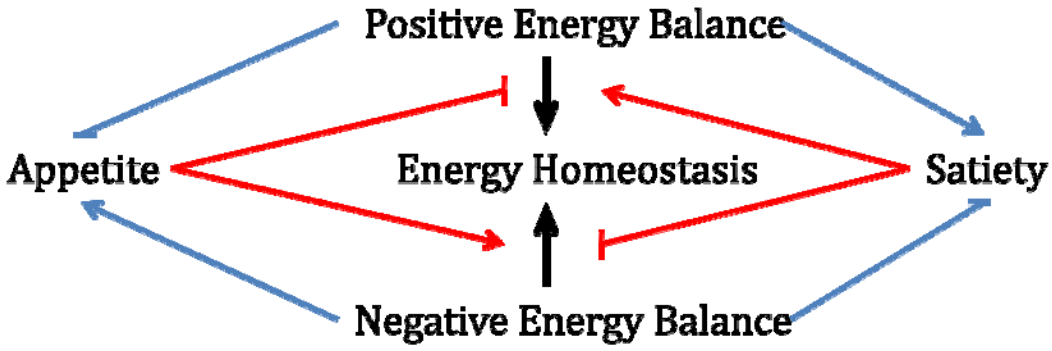

Figure 2- CNS and peripheral maintenance of energy homeostasis. Energy balance is maintained by both the CNS and the endocrine system. Signals originating from the CNS (red arrows) effect behavioral and metabolic changes aimed at stabilizing energy balance (black arrows). Endocrine signals of energy imbalance (blue arrows) target the CNS.

Important regulators of energy balance include the hypothalamic neurotransmitter neuropeptide Y (NPY), the melanocortin pathway in the hypothalamus, and the adipose-derived hormone leptin. Leptin acts as a peripherally derived mediator of energy balance that is released from by white adipose tissue (Friedman and Halaas 1998). Leptin circulates in the bloodstream and crosses the blood-brain barrier to the hypothalamus, where it decreases appetite and increases metabolic rate through inhibition of NPY neurons and activation of proopiomelanocortin (POMC) neurons (Schwartz, Seeley et al. 1996); (Cheung, Clifton et al. 1997); (Balthasar, Coppari et al. 2004).

Appetite and satiety signals originate both peripherally and centrally, ultimately acting on the CNS to stimulate or terminate feeding (Batterham, Heffron et al. 2006). Both signals are intended to maintain energy balance by preventing the starvation or overfeeding of an organism, respectively (Morton, Cummings et al. 2006). Such signals are evolutionarily important precisely because of their roles in maintaining energy balance and, consequently, survival. Stronger appetite signals and weaker satiety signals lead to a positive shift in energy balance, as feeding initiation is more stimulated and cessation signals are weaker.

Obesity resulting from chronic positive energy balance due to altered feeding patterns may be caused by increased appetite signals or decreased satiety signals. Based on a rising trend in obesity across demographics in the United States over the last twenty years, obesity has been declared a national epidemic (Mokdad, Serdula et al. 1999). This thesis aims to address a possible cause of obesity associated with appetite and satiety signaling changes in response to chronic consumption of the monosaccharide fructose, via alteration of hypothalamic sensitivity to the appetite hormone leptin.

Fructose and Satiety

Because of the concurrent increases in high fructose corn syrup (HFCS) use and the prevalence of obesity in the US since 1980, fructose is a possible culprit for the obesity epidemic (Figure 3). The increase in HFCS consumption corresponds to a slight, but not equal, increase in the total consumption of sweeteners, suggesting that an increase in HFCS consumption was at least partially countered by a decrease in the consumption of other sweeteners. If the increase in obesity is not attributable to a significant increase in caloric intake from total sweeteners, then the role of the composition of those sweeteners consumed, which has undoubtedly changed over the last 35 years, must be examined.

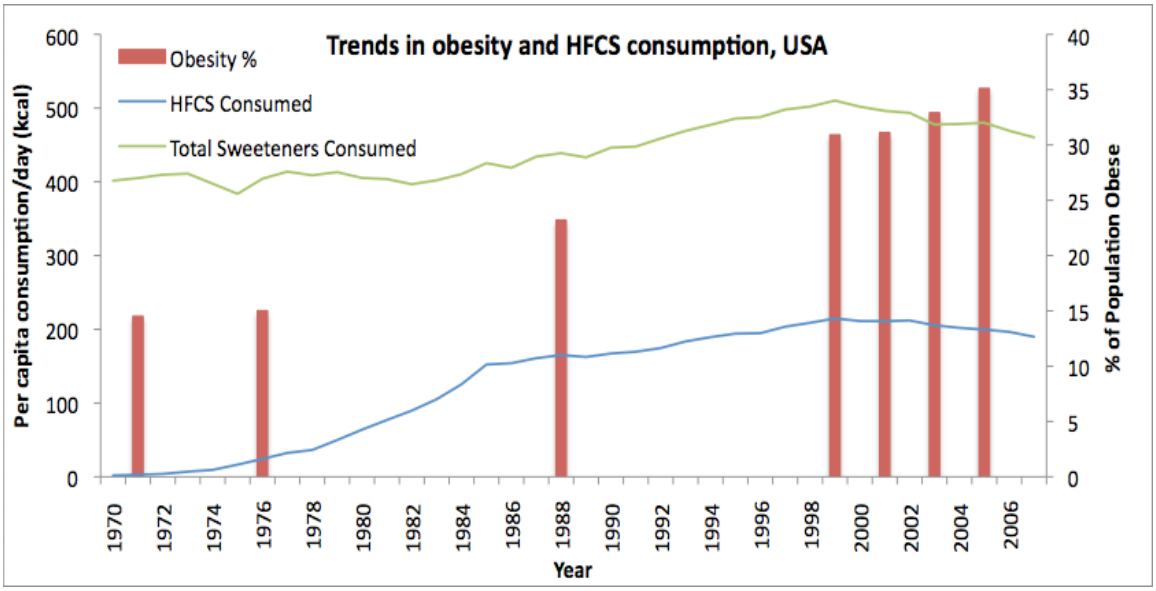

Figure 3- Concurrent rise in the consumption of HFCS and obesity. Obesity is defined as a BMI ≥ 30.0; data used here is from National Health and Nutrition Examination Survey (NHANES), and includes adults aged 20-74 (CDC). Consumed HFCS is the estimated daily number of kcal consumed per capita as reported by the Economic Research Service (USDA). Total sweeteners consumed is recorded as the sum of the daily per capita kcal consumption estimates for the following: 1) refined cane and beet sugar, 2) HFCS, and 3) other sweeteners (USDA). In 1970, kcal of HFCS consumed is equal to 0% of total sweeteners consumed. In 1983, 26% of kcal of total sweeteners was due to HFCS, and in 2007 HFCS was responsible for 41% of the kcal of all sweeteners consumed.

Dietary fructose leads to significantly lower circulating glucose levels and higher triglyceride levels postprandially than dietary glucose (Teff, Elliott et al. 2004). The concentration of glucose affects leptin's activation of POMC neurons in the arcuate nucleus, such that at low glucose levels (90 mg/dL), leptin fails to activate POMC neurons and at high glucose levels (198 mg/dL) leptin activates POMC neurons and produces anorexigenic effects (Ma, Zubcevic et al. 2008). In both mice and rats, central administration of fructose causes increased appetite, whereas central administration of glucose increases satiety (Miller, Martin et al. 2002); (Wolfgang, Cha et al. 2007). Dietary fructose is absorbed into the bloodstream in the gastrointestinal tract, where it is rapidly taken up by the liver and metabolized, whereas glucose is not initially absorbed by the liver (Schaefer, Gleason et al. 2009). This suggests that fructose, because it does not raise circulating glucose levels as much as dietary glucose, does not elicit as strong a leptin-mediated anorexigenic effect via the POMC neurons. This mechanism conceptually supports a link between fructose and decreased satiety, resulting in a positive shift in energy balance.

Chronic consumption of fructose also leads to decreased levels of the gut-derived appetite hormone peptide YY3-36 (PYY) in rats (Lindqvist, Baelemans et al. 2008). PYY binds to NPY receptors in the hypothalamus and prevents the orexigenic effects of NPY (Vrang, Madsen et al. 2006). In addition, fructose, but not glucose, consumption leads to increased circulating levels of the appetite hormone ghrelin in rats (Lindqvist, Baelemans et al. 2008). Ghrelin initiates feeding behavior by activating NPY neurons in the hypothalamus (Mondal, Date et al. 2005). The effect of combined decreased PYY and increased ghrelin resulting from chronic fructose consumption is increased NPY

neuron activation and consequent increased appetite. Paradoxically, however, some studies have shown that consumption of fructose, sucrose, or glucose acutely decreases appetite, as preloading with sweetened beverages leads to decreased caloric intake shortly thereafter (Lindqvist, Baelemans et al. 2008).

Intracellular hypothalamic malonyl-CoA, which is a marker of energy surplus, has anorexigenic effects via upregulation of POMC and downregulation of NPY (Hu, Cha et al. 2003); (Wolf 2006). Central administration of glucose in mice leads to the dephosphorylation and consequent activation of acetyl-CoA carboxylase (ACC), leading to increased concentrations of hypothalamic malonyl-CoA; centrally administered fructose, however, has orexigenic effects mediated by a decrease in hypothalamic malonyl-CoA resulting from phosphorylation and inactivation of ACC, and consequent decrease in hypothalamic malonyl-CoA (Cha, Wolfgang et al. 2008); (Lane and Cha 2009). This further supports the observed role of fructose as an appetite stimulant and satiety-signal suppressant (Figure 4).

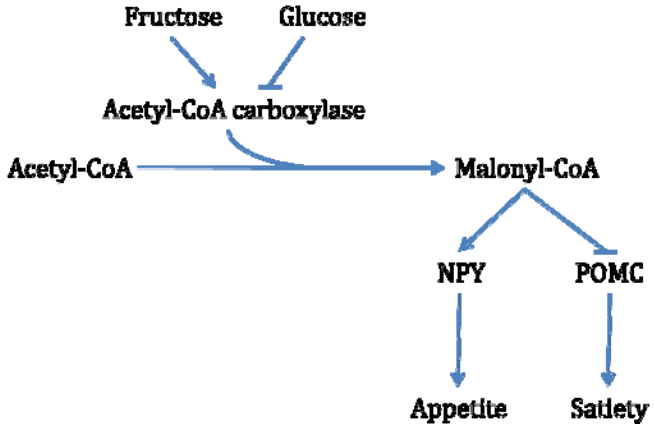

Figure 4- Malonyl-CoA mediated appetite response to central glucose and fructose infusion. Fructose inactivates ACC, decreasing hypothalamic malonyl-CoA, which stimulates NPY release and inhibits POMC release, increasing appetite and decreasing satiety, respectively. Glucose activates ACC, increasing hypothalamic malonyl-CoA, which stimulates POMC release and inhibits NPY release, increasing satiety and decreasing appetite, respectively.

Fructose Metabolism

After absorption from intestinal epithelium to the hepatic portal vein, about 50% of circulating fructose is taken up by the liver (Mendeloff and Weichselbaum 1953). Another 20% of circulating fructose is metabolized by the kidneys, further contributing to the low concentration of circulating fructose after absorption into the bloodstream (Topping and Mayes 1976; Bjorkman and Felig 1982). While the later steps of glycolysis and fructose metabolism are shared, the earlier regulatory steps of glycolysis are absent in fructose metabolism (Mayes 1993).

Fructose and glucose are both transported across cell membranes by GLUT transporters; GLUT2 carries both glucose and fructose into the liver, though the binding of fructose is affected by the glucose concentration, and GLUT5 transports fructose across the blood-brain barrier (BBB). Inside hepatic cells, fructokinase quickly phosphorylates fructose to fructose-1-phosphate (F1P) (Hers 1952). Because fructose is a hexose, it may be also be phosphorylated at C-6 by hexokinase. However, glucose inhibits hexokinase phosphorylation of fructose to fructose-6-phosphate (F6P), and so fructose is phosphorylated by fructokinase to F1P in the presence of glucose (Froesch and Ginsberg 1962). F1P is cleaved into glyceraldehyde and dihydroxyacetone phosphate (DHAP) by aldolase, and glyceraldehyde is subsequently phosphorylated by ATP to form glyceraldehyde-3-phosphate (G3P) in a reaction catalyzed by triokinase (Mayes 1993). G3P and DHAP are common intermediates in glycolysis, and so fructose metabolism shares the remainder of its pathway with glycolysis (Figure 5).

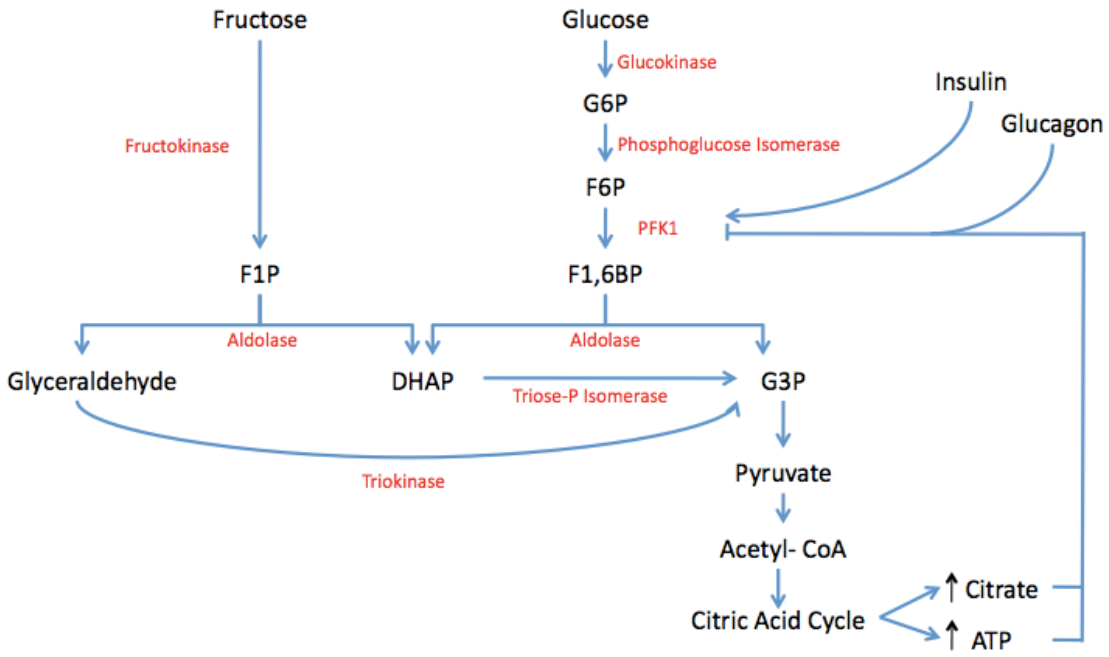

Figure 5- Fructose metabolism and glycolysis. Fructose bypasses the regulatory control of products of the citric acid cycle on PFK1 activity. (G6P, glucose-6-phosphate; F6P, fructose-6-phosphate; F1,6BP, fructose-1,6-bisphosphate; F1P, fructose-1-phosphate; DHAP, dihydroxy-acetone phosphate; G3P, glyceraldehyde-3-phosphate; PFK1, phosphofructokinase-1).

After glucose is transported into hepatic cells by GLUT2, it is phosphorylated by glucokinase to glucose-6-phophate (G6P), which is then isomerized to F6P by phosphoglucose isomerase (Mayes 1993). F6P is then phosphorylated by phosphofructokinase-1 (PFK1) to form fructose-1,6-bis-phosphate (F1,6BP), which is cleaved by aldolase into G3P and DHAP, which is converted into G3P by an isomerase. Each of these molecules of G3P is converted via several steps into pyruvate, which is then dehydrogenated to a molecule of acetyl-CoA (Figure 5). The acetyl-CoA formed from glycolysis may be stored, converted into ketone bodies, or continue to the citric acid cycle to produce NADH, which is necessary for oxidative phosphorylation and ATP synthesis (Mayes 1993).

The main regulatory step in glycolysis is the phosphorylation of F6P to F1,6BP, catalyzed by PFK1 (Underwood and Newsholme 1965). Because the production of glyceraldehyde and DHAP from fructose are not regulated by this same mechanism, fructose absorption in the liver rapidly leads to increased triose intermediates for glycolysis (Mayes 1993). Regulation of PFK1 is related to energy status; products of the citric acid cycle, ATP and citrate, as well as glucagon, an indicator of low blood glucose, inhibit PFK1 activity, whereas the indicator of energetic insufficiency AMP and insulin, which indicates high blood glucose, activate PFK1 (Underwood and Newsholme 1965; Fister, Eigenbrodt et al. 1983). Increased triose intermediates leads to increased flux through to pyruvate and lactate, and more acetyl-CoA, in addition to elevated levels of those intermediates produced by fructose metabolism (Mayes 1993).

An increase in malonyl-CoA is observed in the presence of lactate and pyruvate, so the increase in these intermediates resulting from fructose metabolism likely leads to the increase of lipogenesis (Mcgarry, Takabayashi et al. 1978). The carboxylation of acetyl-CoA is catalyzed by acetyl-CoA carboxylase (ACC), which is activated by insulin, and inhibits a key enzyme in fatty acid oxidation, carnitine palmitoyl transferase 1 (Mcgarry, Leatherman et al. 1978). Thus, elevated malonyl-CoA increases the lipogenic product acyl-CoA, which esterifies with glycerol-3-phosphate, an intermediate that exists in equilibrium with DHAP, to form triglycerides (Mayes 1993). This mechanism of increased lipogenesis associated with fructose is compatible with the observed increases in circulating triglycerides, glucose, and insulin after chronic fructose feeding (Le, Faeh et al. 2006); Figure 6).

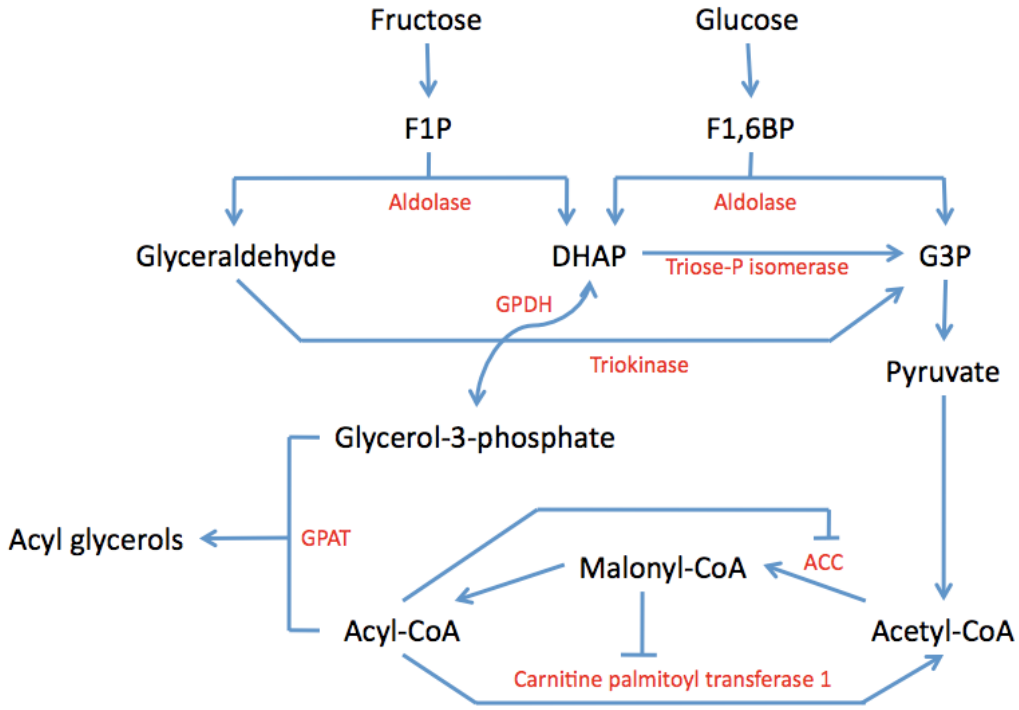

Figure 6- Triglyceride synthesis from products of fructose metabolism and glycolysis. Increase in pyruvate coupled with increased DHAP resulting from fructose metabolism leads to increased triglyceride synthesis in the liver. (Abbreviations: F1,6BP, fructose-1,6-bisphosphate; F1P, fructose-1-phosphate; DHAP, dihydroxyacetone phosphate; G3P, glyceraldehyde-3-phosphate; GPDH, glycerol-3-phosphate dehydrogenase; GPAT, glycerol-3-phosphate acyl transferase).

Leptin and Energy Balance

Leptin is an adipocyte-derived hormone with circulating concentrations directly related to the amount of white adipose tissue (WAT) in mammals (Frederich, Lollmann et

al. 1995). An appetite hormone, leptin exerts anorexigenic effects in the arcuate nucleus of the hypothalamus. Observations of large numbers of mice at Jackson Laboratories in Bar Harbor, ME led to the publication of the first paper regarding the obese gene (*ob*) and an autosomal recessive mutation of *ob* that caused morbid obesity in mice (Ingalls, Dickie et al. 1950). Transfusion of blood from hypothalamic-lesion-induced obese mouse to non-lesioned mice resulted in a negative shift in energy balance associated with decreased food intake, suggesting that the lesioned mice had elevated levels of a circulating factor regulating energy balance (Hervey 1959).

The *ob* gene product was believed to be a circulating factor controlling satiety based on Hervey's parabiosis experiment, assuming hypothalamic action of the protein coded for by the *ob* gene product. Cross-circulation experiments in which *ob* gene product was injected into *ob/ob* mice showed that *ob* was a gene that controlled the expression of a blood-borne anorexigenic factor (Halaas, Gajiwala et al. 1995). After cloning of the *ob* gene and identification of its homolog in humans, it was suggested that the diabetes gene (*db*) encoded the receptor for the *ob* gene product, OB-R (Tartaglia, Dembski et al. 1995).

Homozygous *ob/ob* animals have two copies of a recessive mutation in the gene coding for the cytokine later named leptin, leading to the lack of *ob* RNA and are morbidly obese compared to littermates with normal *ob* expression (Ingalls, Dickie et al. 1950; Zhang, Proenca et al. 1994). *db/db* mice are homozygous for an insertion mutation of 106 nucleotides in the *db* gene coding for the OB-R (leptin receptor), characterized by either early termination of the intracellular region of the leptin receptor or incorporation of the insertion into a novel exon and conservation in the intracellular domain of the

receptor, both of which lead to loss of function (Chen, Charlat et al. 1996). Because *ob/ob* mice have intact leptin receptors but lack leptin, leptin administration to *ob/ob* mice leads to reduction in body weight (Halaas, Boozer et al. 1997). However, *db/db* mice are not sensitive to exogenous leptin because they presumably produce endogenous leptin that has no anorexigenic effect because of the loss of function of the leptin receptor (Halaas, Gajiwala et al. 1995).

After the realization that leptin and its receptor were crucial to the regulation of body weight, leptin was considered to be a component of an energy balance feedback loop involving WAT and the CNS. This feedback circuit begins with an increase or decrease in WAT leading to a corresponding increase or decrease in circulating leptin (Maffei, Fei et al. 1995). As leptin is a signal of energetic sufficiency, higher leptin levels are associated with higher WAT mass. Increased leptin levels are anorexigneic, and the associated decreased food intake leads to decreased WAT and a stabilization of leptin levels, restoring energy balance; decreased leptin levels are orexigenic, increasing appetite and food intake, and hence WAT, leading to the stabilization of leptin levels and energy balance (Friedman and Halaas 1998).

Leptin receptors in the arcuate nucleus are located on two distinct neuronal types in the hypothalamus, increasing expression of neurotransmitters released from POMC neurons and suppressing expression of neurotransmitters released from NPY neurons. Neurotransmitters released from POMC neurons increase satiety, whereas neurotransmitters from NPY neurons increase appetite (Hahn, Breininger et al. 1998; Elmquist 2001); (Hakansson, Brown et al. 1998). Thus, leptin's role in energy balance as a regulator of energy intake has been broadly outlined. However, leptin also possesses a

role in regulation of energy expenditure, making its control of energy balance two-fold (Havel 2000).

Leptin administration in leptin-deficient mice leads to increases in body temperature and physical activity, suggesting a role of leptin in both basal metabolic and behavioral aspects of energy expenditure (Pelleymounter, Cullen et al. 1995). Leptin injections also inhibit circadian drops in metabolic rate in food-restricted mice, suggesting that leptin is important in indicating low energy stores and preventing drops in metabolic rate when food is scarce, but it has less pronounced effects on metabolism when food is abundant (Doring, Schwarzer et al. 1998). Additionally, metabolic rate increases associated with leptin administration involve the inhibition of torpor in rodents, a mechanism for conserving energy during times of scarce food (Geiser, Kortner et al. 1998; Freeman, Lewis et al. 2004). Thus, evolutionarily, leptin seems to prevent drops in metabolic rate when energy stores are low, while simultaneously stimulating hunger in underfed animals. The mechanism by which leptin prevents overeating is likely secondary, and it may fail when constantly high leptin levels, as may occur when food is abundant, cause a decrease in central sensitivity to the effects of leptin, possibly to allow for increased storage of energy.

The Leptin Receptor and Leptin Signaling

After being secreted by white adipose tissue, leptin is carried in the bloodstream to the brain. Leptin, the protein product of the *ob* gene, binds to its receptor, the product of the *db* gene, in the arcuate nucleus of the hypothalamus. Leptin's capacity to regulate energy balance depends on the functionality of leptin receptor, suggesting the importance of a signaling cascade initiated by leptin/leptin receptor interaction. The leptin receptor is

a class I cytokine receptor, meaning it is activated by ligand-induced dimerization, but lacks inherent tyrosine kinase activity (White, Kuropatwinski et al. 1997). The long isoform of the leptin receptor, found predominantly in the hypothalamus, mediates activation of signal transducer and activator of transcription-3 (STAT3) (Baumann, Morella et al. 1996; Banks, Davis et al. 2000).

The receptor proteins have an extracellular leptin-binding domain, and an intracellular domain. Janus kinase 2 (JAK2) is associated with the membrane-proximal region of the receptor's intracellular domain, and is activated by autosphosphorylation upon dimerization of the receptor after leptin-binding (Banks, Davis et al. 2000). JAK2 is important because of its tyrosine kinase activity, as the class of cytokine receptors to which the leptin receptor belongs cannot phosphorylate tyrosine residues (White, Kuropatwinski et al. 1997). The activated JAK2 then phosphorylates two of three tyrosine residues on the intracellular domain of the leptin receptor (Tyr^{985} and Tyr^{1138}). Phosphorylated Tyr^{1138} recruits STAT3, which is tyrosine-phosphorylated by JAK2. JAK2 is normally dephosphorylated by protein tyrosine phosphatase 1B (PTP1B), but, paradoxically, loss of PTP1B leads to elevated leptin signaling, suggesting another role for PTP1B in mediating the response to leptin (Cheng, Uetani et al. 2002). Phosphorylated STAT3 may enter the nucleus, where it regulates the transcription of genes including *socs3*, which encodes suppressor of cytokine signaling-3 (SOCS3), which blocks leptin signal transduction by inhibiting JAK2 activity and by binding phosphorylated Tyr^{985} (Figure 7; (Bjorbaek, Lavery et al. 2000; Munzberg, Huo et al. 2003).

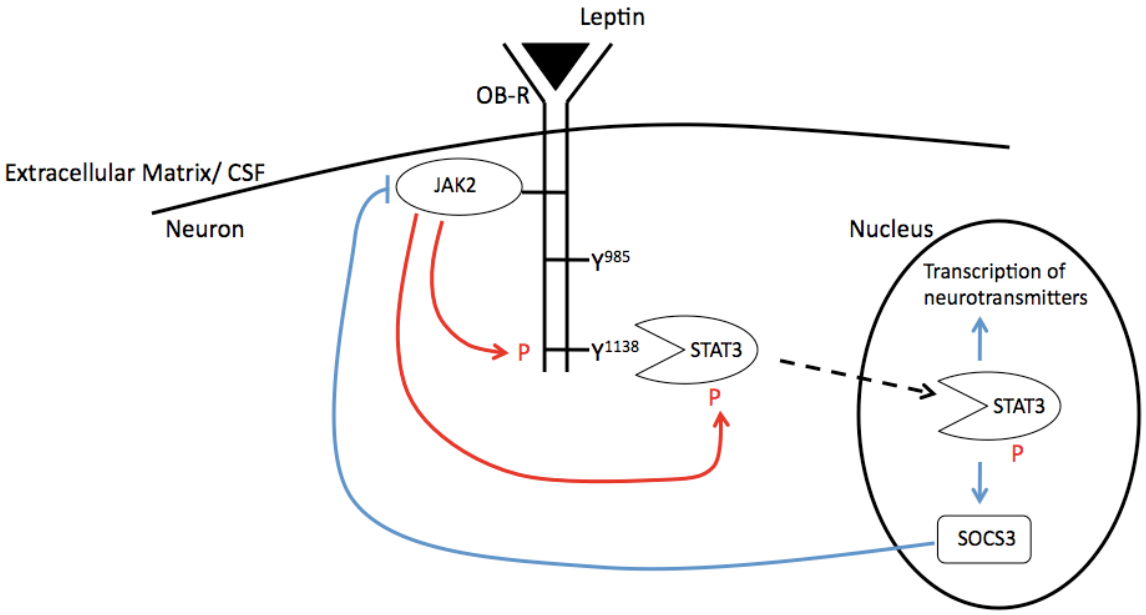

Figure 7- An overview of JAK/STAT leptin signaling in neurons. Leptin (black triangle) association causes dimerization of its receptor, OB-R, which activates JAK2. JAK2 phosphorylates tyrosine residue 1138, which recruits STAT3. JAK2 then phosphorylates STAT3, which translocates to the nucleus, where it activates *socs3* transcription, which codes for an inhibitor of JAK2, and regulates transcription of neurotransmitters (increasing α-MSH transcription, and decreasing GABA, NPY, and AgRP transcription). Red lines indicate JAK2 phosphorylation, blue lines indicate regulation.

The Arcuate Nucleus

Energy balance is regulated by the CNS largely via the arcuate nucleus in the hypothalamus. The arcuate nucleus is an aggregation of neurons important in the maintenance of energy balance. Leptin binds to its receptor in two distinct neuronal types in the arcuate nucleus of the hypothalamus, POMC neurons and NPY neurons (Friedman and Halaas 1998). The circuitry of these neurons in the arcuate nucleus underlies leptin's control over energy intake and expenditure.

In the hypothalamus, leptin receptors are transmembrane receptors on POMC neurons and NPY neurons. Leptin's association with its receptor on POMC neurons increases expression of the anorexigenic peptide α-melanocyte stimulating hormone (α-MSH) via the JAK/STAT pathway, and binding on NPY neurons lead to decreased expression of the inhibitory neurotransmitter γ-Aminobutyric acid (GABA) and the orexigenic peptides agouti-related protein (AGrP) and NPY (Schwartz, Seeley et al. 1996; Hahn, Breininger et al. 1998; Sahu 2003; Balthasar, Coppari et al. 2004).

POMC neuronal firing is activated by leptin, causing the release of α-MSH which subsequently binds to the melanocortin-4 receptor (MC4R) (Odonohue and Dorsa 1982; Cheung, Clifton et al. 1997; Cowley, Smart et al. 2001). The anorexigenic effect attributed to POMC neurons is due to the interaction of α-MSH with the MC4R, the deletion of which causes obesity decreased food intake and obesity (Huszar, Lynch et al. 1997; Farooqi, Yeo et al. 2000). Leptin also increases POMC neuron action potential frequency both by affecting depolarization directly and by reducing the inhibitory effects of adjacent NPY neurons which release GABA (Cowley, Smart et al. 2001). Leptin receptors on POMC neurons are critical to leptin's regulatory role in body weight, as their deletion leads to mice with elevated body weight (Balthasar, Coppari et al. 2004)

When leptin binds to its receptor on NPY neurons, it inhibits the release of NPY, AgRP, and GABA (Jang, Mistry et al. 2000; Cowley, Smart et al. 2001; Morrison, Morton et al. 2005). Both NPY and GABA elicit an orexigenic effect by inhibiting POMC neurons, an effect which is enhanced when leptin is not present (Roseberry, Liu et al. 2004). The role of NPY in stimulating appetite and reducing satiety has been demonstrated in *ob/ob* mice, with loss of NPY resulting in attenuation of obesity in the

leptin-deficient mice (Erickson, Hollopeter et al. 1996). While NPY is an agonist for NPY Y1 and Y5 receptors that regulate feeding, both NPY and AgRP act by antagonizing MC4R, decreasing the satiety signal of the melanocortin pathway (Ollmann, Wilson et al. 1997; Hahn, Breininger et al. 1998; Hansen and Morris 2002). NPY neuron action potential frequency was increased by fasting in leptin-sensitive mice, and the frequency decreased after leptin injection, suggesting that leptin decreases the orexigenic effect of NPY neurons by causing electrophysiological changes (Takahashi and Cone 2005). Thus, leptin signaling in the arcuate nucleus suggests a complex circuit between orexigenic and anorexigenic hormones (figure 8).

In summary, the net effect of leptin is to increase satiety by increasing α-MSH binding to MC4R in target cells and to decrease appetite by decreasing NPY binding to NPY 1/5 receptors in target cells.

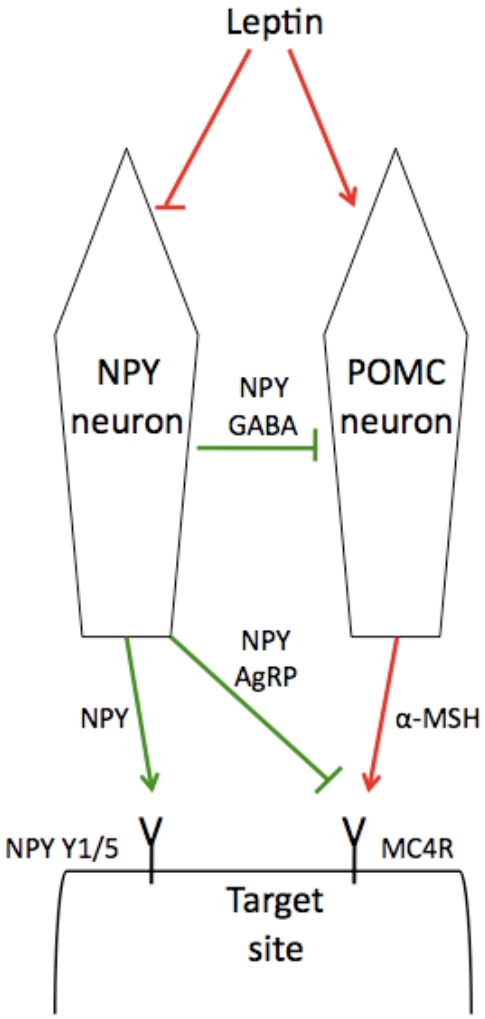

Figure 8- Effects of leptin in the hypothalamic arcuate nucleus circuit regulating energy balance. Leptin activates POMC neurons, which releases α-MSH, stimulating the anorexigenic MC4R. Leptin also decreases the activity of NPY neurons, which normally 1) inhibit PMC neurons through the release of NPY and GABA, 2) inhibit melanocortin signaling by antagonizing MC4R, and 3) stimulate appetite through NPY association with orexigenic NPY Y1 or Y5. Red lines indicates anorexigenic regulation and green lines indicates orexigenic regulation in the arcuate nucleus.

Leptin Resistance

When elevated leptin levels do not produce an anorexigenic response, an animal is said to be leptin resistant (Myers, Cowley et al. 2008). While several conditions, notably chronic high caloric intake, may cause leptin resistance, the loss of sensitivity itself predisposes an animal to increased weight gain due to disrupted energy balance regulation. Such an interruption may lead to a cycle of constant positive shifts in energy balance, as decreased sensitivity leads to overeating and obesity, which in turns contributes to further losses in leptin sensitivity, and so forth (Zhang and Scarpace 2006). In this thesis, I will measure the leptin sensitivity of mice exposed to a high-fructose diet.

Experimentally, leptin sensitivity is determined by responsiveness to exogenously administered leptin. Injections, either interperitoneal (IP) or intracerebroventricular (icv), are meant to mimic increases in endogenous leptin in a more controlled manner so that a hypothesized response to the leptin may be observed. For leptin-sensitive animals, a leptin injection causes acute decrease in body weight and food intake, whereas leptin-resistant animals do not respond to a leptin injection any differently than they would to a saline injection. Differences in leptin signaling are measurable via assays for phosphorylated-STAT3 (p-STAT3), which show decreased p-STAT3 levels in leptin resistant animals (Vasselli, Johnson et al. 2002).

Diet-induced obesity is a well-studied culprit for leptin resistance. High fat diets decrease leptin receptor expression as well as leptin signaling in the hypothalamus (Zhang and Scarpace 2006). The strongest resistance appears to be to peripheral leptin, as diet-induced obese mice are resistant to IP injections of leptin, but relatively sensitive to leptin administered icv (VanHeek, Compton et al. 1997; Lin, Thomas et al. 2000;

Munzberg, Flier et al. 2004). The central sensitivity to leptin in peripherally leptin resistant animals suggests that the transport of leptin across the blood-brain barrier (BBB) may be impaired. However, with peripheral leptin resistance, central leptin sensitivity may also be attenuated such that p-STAT3 levels are decreased below control animals after both central and peripheral leptin injections, suggesting that impaired leptin transport occurs in conjunction with decreased signaling upstream of STAT3 phosphorylation (El-Haschimi, Pierroz et al. 2000). Interestingly, diminished SOCS3 expression associated with haploinsufficiency of *socs3* leads to increased leptin sensitivity and attenuation of weight gain in diet-induced obesity, suggesting that suppression of signaling is important in leptin sensitivity (Howard, Cave et al. 2004).

In diet-induced leptin resistance, circulating leptin levels are elevated above those of control-fed leptin-sensitive mice, and the weight gain and increase in fat mass of these mice despite their high leptin levels is also characteristic of leptin resistance (Zhang and Scarpace 2006). Elevated central leptin concentrations also contribute to leptin resistance, further indicating that not all leptin resistance is due to diminished transport of leptin across the BBB into the cerebrospinal fluid (Zhang and Scarpace 2006). In small rodents, the elevated leptin levels associated with leptin resistance also inhibit torpor, which may be entered normally upon restoration of lower circulating leptin levels (Geiser, Kortner et al. 1998; Freeman, Lewis et al. 2004). Based on the susceptibility of leptin resistant animals to diet-induced obesity, the reversal of leptin resistance is a fundamental step in controlling diet-induced obesity and restoring energy balance to animals; one method for increasing the leptin sensitivity of mice is caloric restriction,

leading to a reversal of diet-induced leptin resistance via increases in leptin signaling (Scarpace and Zhang 2009).

Caloric Restriction

Limiting the food intake of animals obviously leads to a negative shift in energy balance, as energy intake drops while energy expenditure is not directly mediated by caloric intake. Important physiological changes occur when an animal is calorically restricted, including reduced circulating leptin levels and increased leptin signaling, such that caloric restriction successfully reverses leptin receptor expression and signaling defects associated with leptin resistance (Wilsey and Scarpace 2003; Wilsey and Scarpace 2004; Zhang and Scarpace 2006).

Caloric restriction also leads to torpor in mice, as torpor requires decreased energy expenditure to compensate for decreased energy intake (Overton and Williams 2004). However, caloric restriction leads to torpor only in leptin sensitive mice with low circulating leptin levels (Gavrilova, Leon et al. 1999). Because of this, caloric restriction may be expected to both reverse leptin resistance and permit entry into torpor in animals with diet-induced alterations of leptin sensitivity.

Fructose and Leptin Sensitivity

In rats, chronic fructose feeding causes leptin resistance and amplifies weight gain in response to subsequent high-fat feeding (Shapiro, Mu et al. 2008). Shapiro et al. fed rats a 60% fructose diet versus a fructose-free control for six months and then tested for leptin resistance. Half of the rats from each group were then switched to either a high fat diet or maintained on their initial diet. After six months of high-fructose feeding, rats had

decreased sensitivity to peripherally administered exogenous leptin (Figure 9). Rats maintained on a high-fructose diet failed to respond with decreased food intake upon IP injection of leptin compared to saline injection, whereas fructose-free fed rats decreased their food intake significantly after IP injection of leptin compared to saline injection. The finding that high fructose fed rats had lower levels of p-STAT3 indicates either that the interruption in the leptin signaling pathway occurred prior to tyrosine phosphorylation of STAT3 by JAK2, or that the fructose-fed rats had reduced expression of STAT3.

Because neither circulating levels of leptin nor expression of the leptin receptor differed between the feeding groups, the altered signaling cannot be attributed to fructose alteration of expression of either the *ob* or the *db* gene (Shapiro, Mu et al. 2008). Thus, the interruption in leptin signaling, at the leptin receptor, could be due to any of a number of causes: 1) lack of interaction between leptin and its receptor, 2) failure of the interaction to activate JAK2, 3) failure of activated JAK2 to phosphorylate the tyrosine residue that recruits STAT3, 4) failure of the phosphorylated tyrosine residue to recruit STAT3 for phosphorylation, or 5) failure of JAK2 to phosphorylate recruited STAT3.

Shapiro et al. also reported that high-fructose feeding led to increased triglyceride levels compared to control fed rats (control, 143.3±12.3 mg/dl; high fructose, 280.7±32.0 mg/dl; $P < 0.0001$) (Shapiro, Mu et al. 2008). Notably, triglycerides have been shown to inhibit the transport of leptin across the BBB, which suggests that the elevated triglycerides associated with fructose-induced leptin resistance cause decreased leptin sensitivity because of diminished leptin-leptin receptor interactions due to lower leptin levels in the cerebrospinal fluid (Banks, Coon et al. 2004). In the context of fructose-

induced elevated triglyceride levels, this mechanism for leptin resistance makes sense, but has yet to be tested directly.

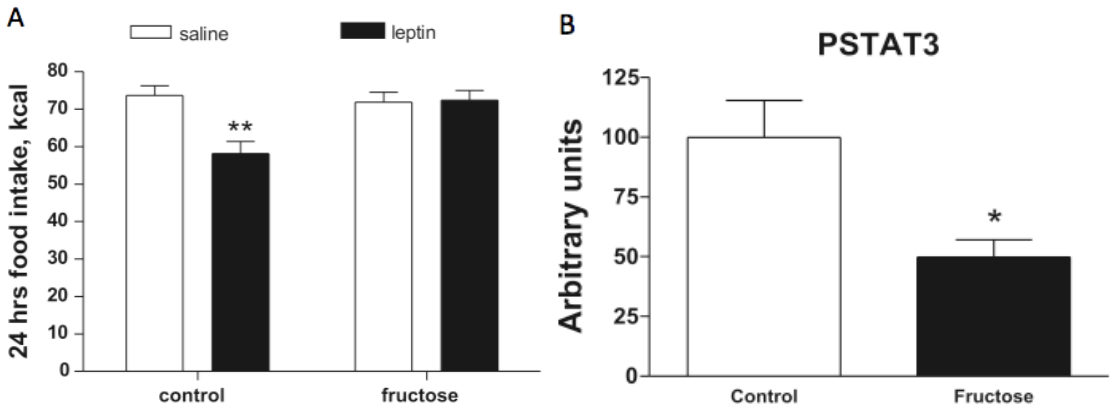

Figure 9- Effects of fructose feeding on leptin sensitivity and signaling from (Shapiro, Mu et al. 2008). *A*) After 6 months on a high fructose diet, rats were resistant to anorexigneic effect of peripherally administered exogenous leptin as gauged by food intake over the 24 hours after an injection with either leptin or saline (**$P < 0.04$ for injections of saline vs. leptin in control fed rats). *B*) Analysis of hypothalamic levels of PSTAT3, a downstream marker of leptin signaling, showed a significant decrease in phosphorylated STAT3 in fructose-fed leptin resistant rats (*$P = 0.015$)

Hypotheses

Since the 1980s, the prevalence of obesity in humans in the United States has increased dramatically, concurrent with a rise in American consumption of HFCS. In this experiment we are examining the effects of a chronic high fructose diet on the ability of mice to regulate their energy balance via the adipocyte-derived hormone leptin.

I hypothesize that fructose-feeding will lead to a positive shift in energy balance, manifest as increased body weight due either to increased energy intake or decreased energy expenditure.

I propose induced leptin resistance as the mechanism by which high-fructose-feeding induces a positive shift in energy balance. I hypothesize that a chronic high fructose diet will decrease peripheral sensitivity to leptin in mice, observable as attenuated response to IP injections of recombinant murine leptin in terms of food intake and body weight. One possible mechanism for this is increased circulating triglycerides associated with the metabolism of fructose, which may decrease the permeability of the BBB to leptin.

I further hypothesize that caloric restriction will alleviate the developed resistance to leptin, either with or without removal from the fructose diet. Treatment aimed at the reversal of leptin resistance resulting from fructose overconsumption has obvious clinical applications, particularly given the dramatic rise in the consumption of HFCS over the last three decades.

MATERIALS AND METHODS

Experiment 1

Animals

Upon arrival, 21 male Hsd:NSA(CF-1) mice purchased from Harlan Laboratories, aged 6-8 weeks, were given ad libitum chow (Teklad) and housed at 23°C in the animal care facility at Williams College. Mice were housed individually with a 12:12-hour light-dark cycle (4:00 on, 16:00 off). Procedures and experimental protocols were approved by the Williams College Institutional Animal Care and Use Committee.

Experimental Diets

Mice were randomly divided into feeding groups (control: n=11, experimental: n=10). The experimental group was given free access to a 60% fructose diet and the control group was allowed free access to a fructose-free diet. Both diets were made in lab and were delivered daily to mice in the animal facility. Feeding and body weight measurements were done in the hour before the start of the dark phase.

Both diets (table 1) were dry mixtures to which water was added immediately prior to delivery to the animals, necessitating both daily changing of food and quantitative correction to account for dehydration of the food. The weight of the high-fructose food that was given to each mouse daily (9 g wet) was 8 g dry mixture and 1 g H_2O, and the weight of the control food given daily to each mouse (13 g wet) was 8 g dry mixture and 5 g H_2O. Different proportions of water were needed because of the difference in the source of carbohydrates in the diets.

Table 1- Ingredients for high-fructose diet and control fructose-free diets: both dry diets were made weekly and kept at 4°C until use, when water was added to daily portions of each.

Control Diet			**High-fructose Diet**	
Ingredient	**g/kg diet**		**Ingredient**	**g/kg diet**
Corn starch	600		Fructose	600
Casein	207		Casein	207
DL-methionine	3		DL-methionine	3
Celufil	79.96		Celufil	79.96
Rogers-Harper Mineral Mix	50		Rogers-Harper Mineral Mix	50
Teklad Vitamin Mix	10		Teklad Vitamin Mix	10
Zinc Carbonate	0.04		Zinc Carbonate	0.04
Lard	15.8		Lard	15.8
Corn Oil	34.2		Corn Oil	34.2

Metabolic Rate Measurement

After 10 weeks on their respective diets, a subset of mice from each feeding group (fructose, n=3; control, n=2) were housed for 24 hours in a closed, airflow-regulated cage at 18°C and metabolic rate was measured by indirect calorimetry. Oxygen consumption, room oxygen and carbon dioxide concentration, flow rate through cages, ambient temperature, and room pressure were monitored to calculate metabolic rate in mL O_2/min.

Flow through the cages was driven by an electrical pump (Barnant Co.), and was controlled by flow regulators allowing 250-350 mL/room air/minute. Humidity of air entering and leaving the cages was 0%, maintained by drying columns (Drierite). Oxygen and carbon dioxide content of the selected channel were measured by gas analyzers (AEI Tech.), which monitored the three animal cages and the room air, regularly switching between them using a channel switcher Qubit Systems).

Leptin Responsiveness

Recombinant murine leptin (ProSpec Bio), reconstituted in saline solution, was administered to randomly chosen mice after 3 weeks of either high-fructose (n=10) or control (n=11) diet interperitoneally at a dose of 5 mg/kg. Saline was injected as vehicle control in the remainder of the high-fructose-fed (n=10) or control-fed (n=11) mice. Injections took place in the hour before the start of the dark phase. As a positive control for the efficacy of the leptin solution, 6 male C57Bl/6 mice were calorically restricted for 3 days, and then injected either with saline (n=3) or leptin, 5 mg/kg (n=3). Food intake and body weight was recorded 6, 24, and 48 hours after injections.

Tissue and Blood Harvesting

After examining metabolic profiles, all mice were sacrificed under isofluorane anesthesia and 0.4-0.8 mL of blood was collected by cardiac puncture, and immediately spun in a microcentrifuge to isolate plasma, which was immediately frozen at -80°C for later analysis. Hearts and livers were removed and immediately frozen at -80°C, where they were stored for later analysis.

Plasma Biomarkers

Plasma leptin levels were measured by ELISA (Millipore). Two ELISAs were run because the samples were too concentrated for the standard curve and were consequently diluted ten-fold for the second ELISA.

Data Analysis

Data are reported as mean ± standard error. Body weight averages and average cumulative food intake for mice across feeding groups were compared using the unpaired

student's t-test. Average food intake following leptin or saline injections was compared using a one-way ANOVA followed by post hoc LSD tests.

Need for Experiment 2

Food intake measurements in Experiment 1 were imprecise (see *Discussion* below). In addition, the Hsd:NSA(CF-1) mice were likely too large to enter torpor when they were fasted at cool temperatures. To address these concerns, a second experiment was conducted in which pelleted food was used to increase the accuracy of the food intake data, and smaller C57Bl/6 mice were used.

Experiment 2

Animals

Pregnant C57Bl/6 females were purchased from Harlan. Upon delivery, litters were housed with mothers for 4 weeks. At 4 weeks of age, 38 males were moved to individual housing at 23°C and weaned directly to randomly assigned experimental diets. Mice were housed individually in the animal care facility at Williams College on a 12:12-hour light-dark cycle (0:00 to 12:00). All procedures and experimental protocols were approved by the Williams College Institutional Animal Care and Use Committee.

Experimental Diets

Mice were randomly divided into feeding groups (n=19 for each). The experimental group was given free access to a 60% fructose diet (Harlan Teklad, TD.89247; 5.2% fat, 60.4% fructose, 18.8% protein, 3.6 kcal/g). The control group was allowed free access to a fructose-free diet (Harlan Teklad, TD.05075; 5.2% fat, 60% corn starch, 18.3% protein, 3.6 kcal/g). Food intake was measured every Monday through

Friday, and body weight was measured twice weekly, in the hour before the start of the dark phase.

Metabolic Rate Measurements

In weeks 2, 4, 7, 9, 11, and 14, seven animals from each feeding group were housed for 24 hours in a closed, flow-regulated cage and metabolic rate was measured by indirect calorimetry. The same seven animals were used each time metabolic rate was measured. Oxygen consumption, room oxygen and carbon dioxide concentration, flow rate through cages, ambient temperature, and room pressure were monitored to calculate metabolic rate in mL O_2 / min. The respiratory exchange ratio (RER) was calculated and the metabolic rate was converted to kcal / hour, which allowed us to compare energy intake as kcal consumed to energy expenditure.

Metabolic setup was the same as described for Experiment 1, but with 7 cages in series in addition to the room air monitor.

Leptin Responsiveness

Recombinant murine leptin (R & D Systems), reconstituted in 20 mM Tris-HCl, pH 8, was administered to randomly chosen mice after 14 weeks of either high-fructose (n=9) or control (n=10) diet interperitoneally at a dose of 5 mg/kg. As vehicle control, 20 mM Tris-HCl, pH 8, was injected IP in the remainder of the high-fructose-fed (n=10) or control-fed (n=9) mice. Injections took place in the hour before the start of the dark phase. Food intake and body weight was recorded 6, 24, and 48 hours after injections.

Tissue and Blood Harvesting

After testing leptin sensitivity, 24 mice (n=12 from each feeding group) were randomly selected and fasted for 24 hours. One hour before the start of the dark phase, these fasted mice and twelve randomly selected fed mice were sacrificed under isofluorane anesthesia and blood was collected by cardiac puncture and immediately frozen at -80°C. Hearts and livers were removed and immediately frozen at -80°C.

Plasma Biomarkers

Plasma glucose and triglyceride levels were assayed using kits and provided instructions from Wako USA, and non-esterified fatty acid (NEFA) levels were assayed using materials and provided instructions from Stanbio Lab.

Data Analysis

Data are reported as mean ± standard error. Body weight averages and cumulative food intake for mice across feeding groups were compared by repeated measures analysis. Average metabolic rates were compared across feeding groups by one-way ANOVA followed by post hoc LSD test. Average food intake following leptin or saline injections was compared by one-way ANOVA followed by post hoc LSD test.

RESULTS

Experiment 1

Caloric Intake

Over the course of the 3 weeks, food intake was measured 5 times weekly. After 1.5 weeks, mice on the high fructose diet had consumed significantly fewer calories cumulatively (185.9 ± 4.0 kcal) than mice on the control diet (205.5 ± 7.1 kcal), and after 3 weeks, mice on the high fructose diet had consumed significantly fewer calories cumulatively (326.7 ± 6.4 kcal) than mice on the control diet (356.6 ± 11.2 kcal) (Figure 10).

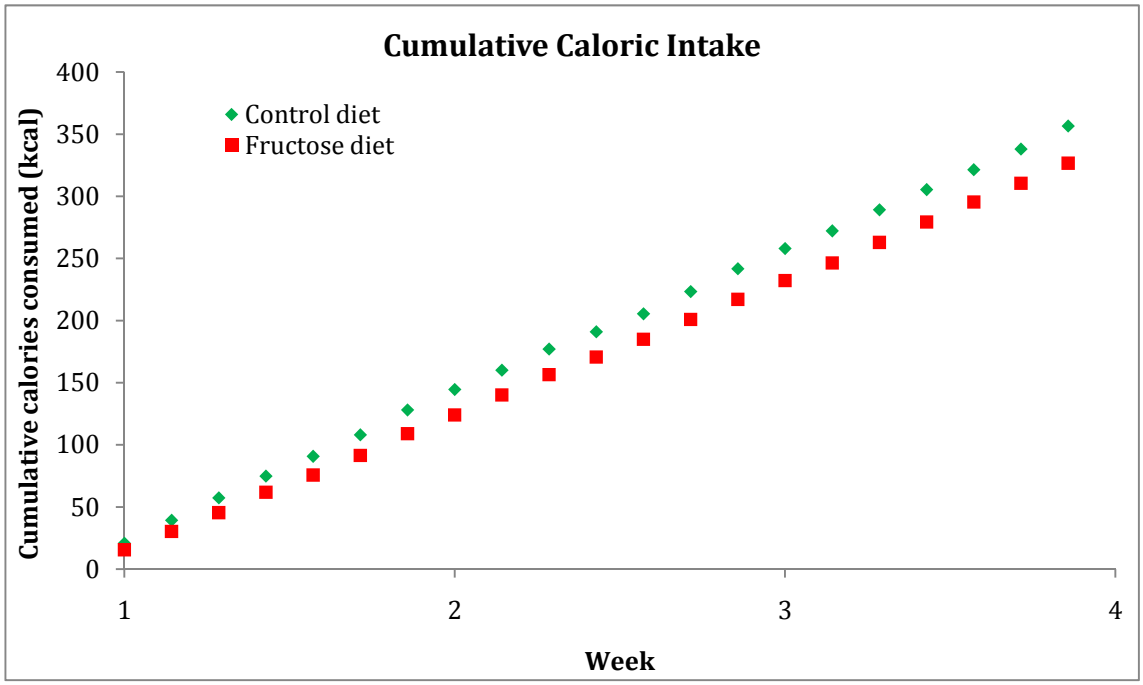

Figure 10- Average cumulative caloric intake for mice in the control-feeding group (n=11) was higher than in the fructose-feeding group (n=10) over the course of the 3 weeks ($p<0.05$ for all days measured).

Body Weight

Over the course of the 6 weeks, body weight was measured 2 times weekly. At the beginning of the study, the mice that were subsequently given the high fructose diet (35.4 ± 0.4 g) and the control diet (35.4 ± 0.4 g) had similar body weights. After 3 weeks, mice on the high fructose diet (37.2 ± 0.5 g) did not have significantly different body weights than mice on the control diet (36.0 ± 0.6 g). In addition, after 5.5 weeks, mice on the high fructose diet (39.1 ± 0.8 g) did not have body weights that significantly differed from mice on the control diet (37.7 ± 0.6 g) (Figure 11).

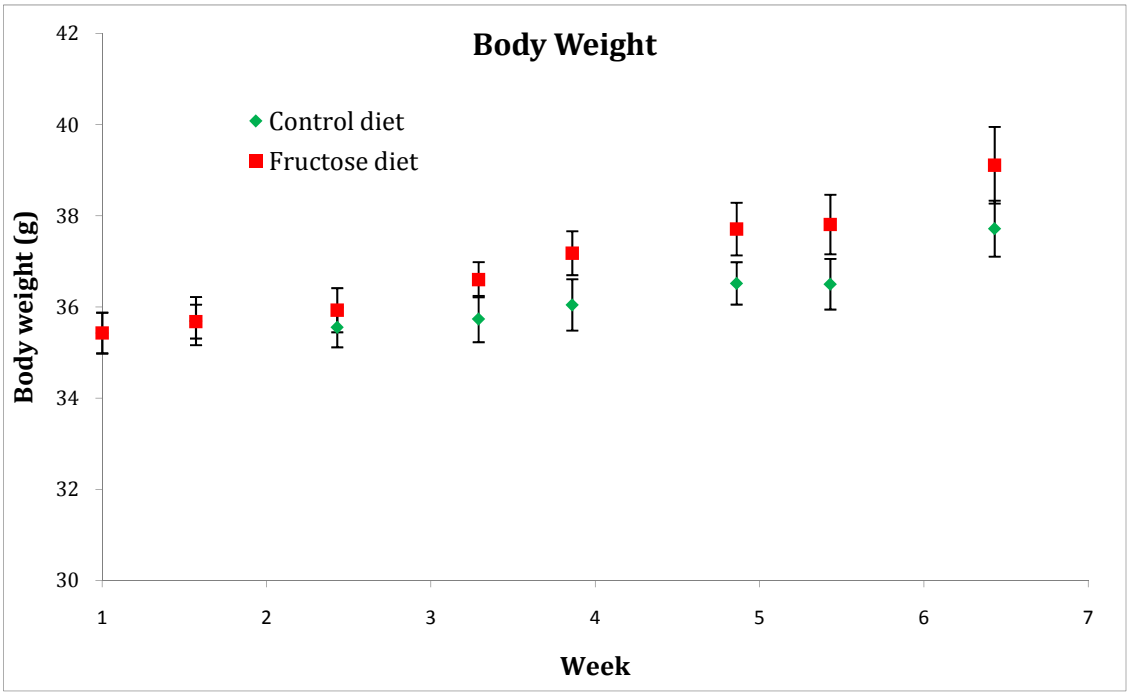

Figure 11- Over the course of the 6 weeks on the diet, there was no significant difference in average body weight between control-fed mice and high-fructose-fed mice.

Leptin Sensitivity

After 3 weeks on their respective diets, animals were injected with leptin or saline vehicle. Leptin administration did not significantly decrease food intake over any interval compared to saline injection, for either diet (Figure 12). While body weight was depressed after leptin administration to control-fed mice (Figure 13), the starting body weights differed as well. There is no difference in percent change in body weight from the time of leptin administration compared to saline in either feeding group (figure 14). Increased body weight in all groups over the first 6 hours after injection is due to feeding at the start of the dark phase. The Hsd:NSA(CF-1) mice that were injected with leptin did not show a significant decrease in food intake compared to saline-injected mice, nor were their body weights lower after injection.

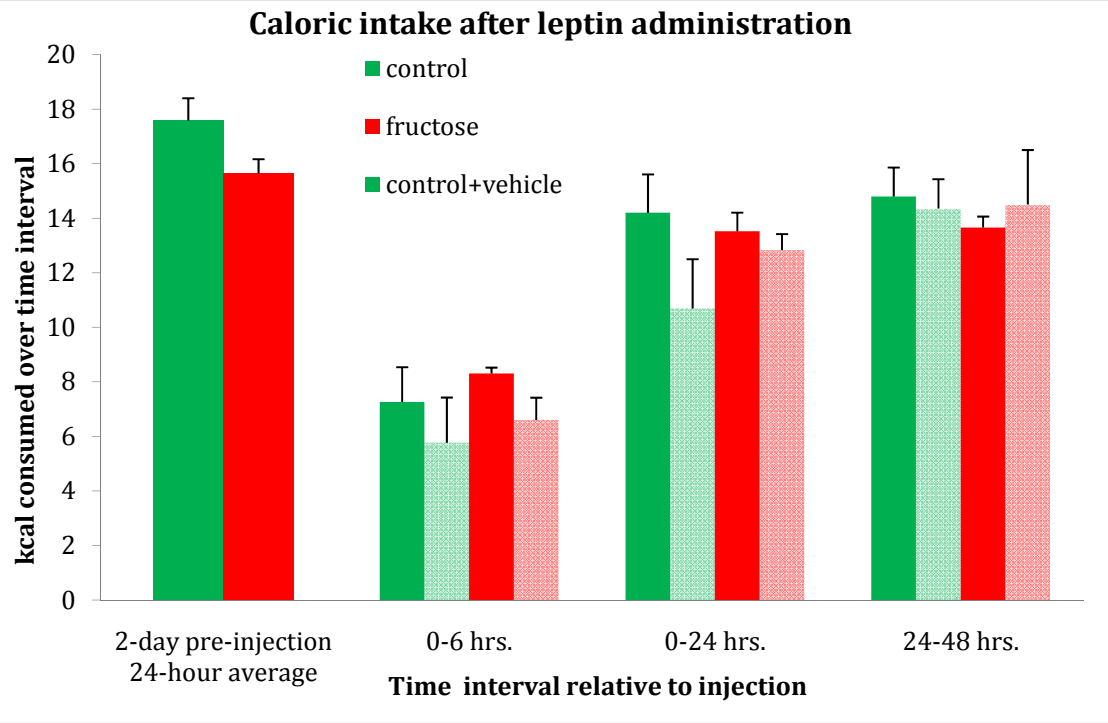

Figure 12- Caloric intake after leptin administration is not different from saline administration on either diet. Therefore, leptin administration failed to produce an anorexigenic effect in either the high-fructose-fed or the control-fed mice.

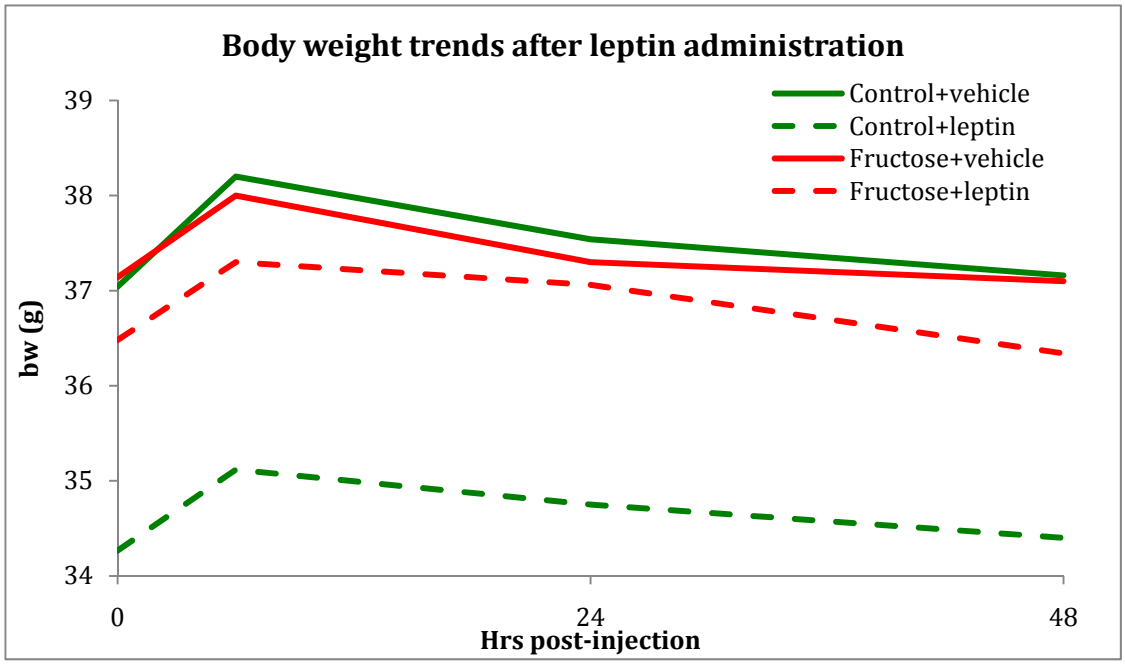

Figure 13- Body weights for each experimental group in the 48 hours post-injection.

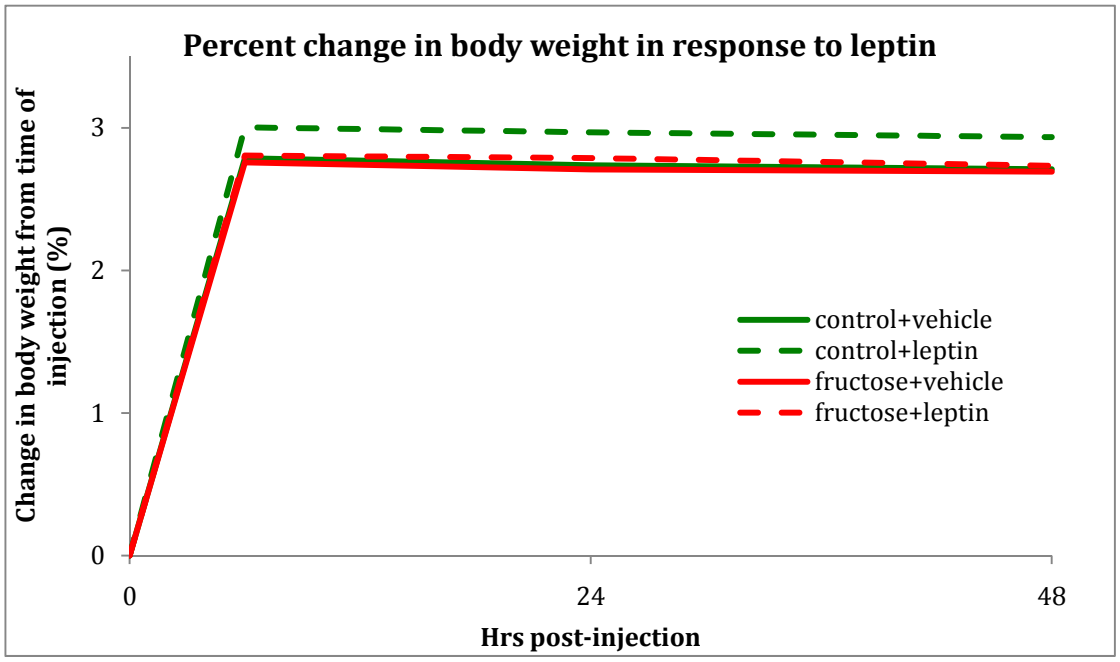

Figure 14- Body weight percent changes for each experimental group in the 48 hours post-injection. All feeding groups increased in body weight by almost 3 percent in the 6 hours after injection, and no statistically significant differences were observed over the 48 hours after leptin or saline administration.

Metabolic Rate and Energy Balance

When fasted at 18°C, there was no difference in metabolic rate between control-fed and high-fructose-fed mice (Figure 15). Neither high-fructose-fed nor control-fed mice entered torpor throughout over the course of the 24-hour fast.

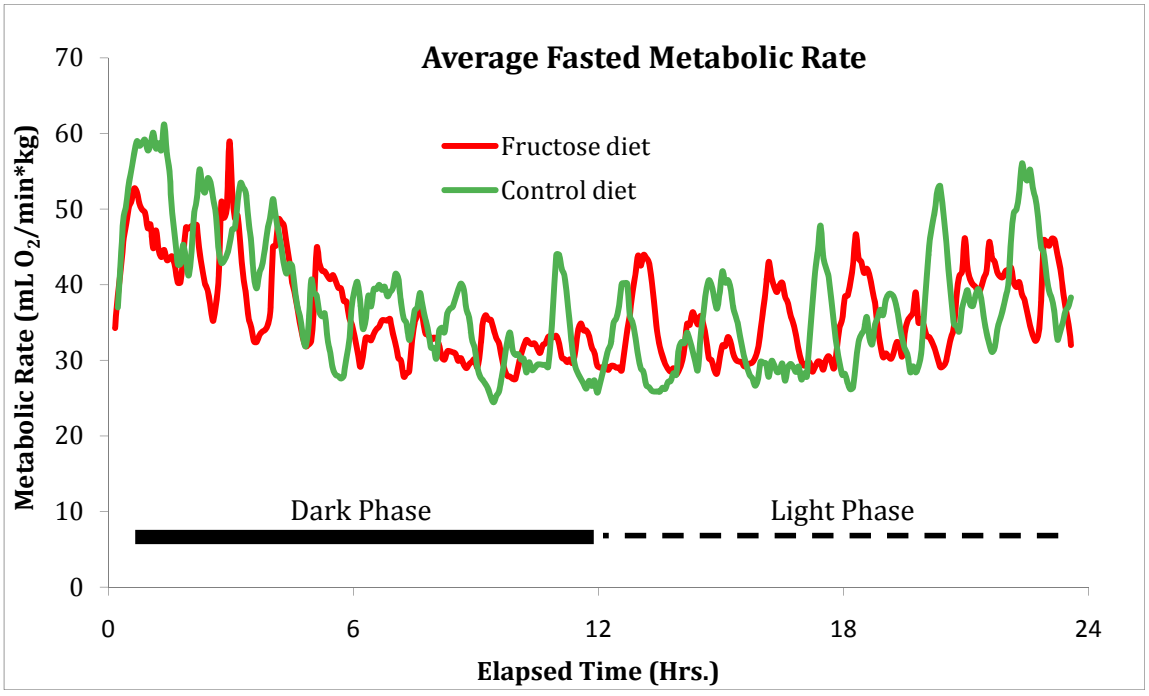

Figure 15- Neither control-fed nor high-fructose-fed mice enter torpor when fasted at 18°C. Additionally, metabolic rate of control-fed mice (n=2) is similar to that of high-fructose-fed mice (n=3) when fasted at cool temperatures.

Plasma Biomarkers

The first undiluted ELISA showed no significant difference in plasma leptin levels between mice fed on the control (24.4 ± 3.2 ng/mL) or high-fructose (22.9 ± 6.3 ng/mL) diets, likely because all levels were near the upper limit of the standard curve. The variability was greatly increased after diluting the plasma samples, although the

second ELISA still showed no difference between control-fed (33.7 ± 21.1 ng/mL) or high-fructose-fed (64.7 ± 40.5 ng/mL) mice.

Experiment 2

Rationale for Second Experiment

Because of inherent imprecise administration of diets and the impossibility of accurately measuring food intake, I conducted a second experiment with a pre-manufactured, pelleted diet to increase the accuracy of my energy intake measurements, which is important given the addition of the comparison of energy intake to energy expenditure in Experiment 2.

Additionally, the strain of mouse used for experiment 1, Hsd:NSA(CF-1), is larger than C57Bl/6 mice, which may have played a role in the failure of even the control-fed mice in Experiment 1 to enter torpor.

Caloric Intake

Over the course of the 14 weeks, food intake was measured 5 times weekly. After 7 weeks, mice on the high fructose diet (596.5 ± 5.0 kcal) had consumed significantly more calories cumulatively than mice on the control diet (526 ± 4.4 kcal), and after 14 weeks, mice on the high fructose diet (1206.6 ± 9.1 kcal) consumed significantly more calories cumulatively than mice on the control diet (1098.0 ± 8.5 kcal) (Figure 16). Notably, these results differ from the caloric intake data from Experiment 1, in which we found control-fed mice were consuming more calories than fructose-fed mice.

The difference in cumulative kcal consumed between fructose-fed and control-fed mice grew each week, indicating that mice on the high-fructose diet were consistently

eating more than mice on the control diet. The average difference in daily calories consumed during weeks 1 and 2 was greater (2.17 ± 0.16 kcal) than the difference during weeks 9 and 10 (0.86 ± 0.19 kcal).

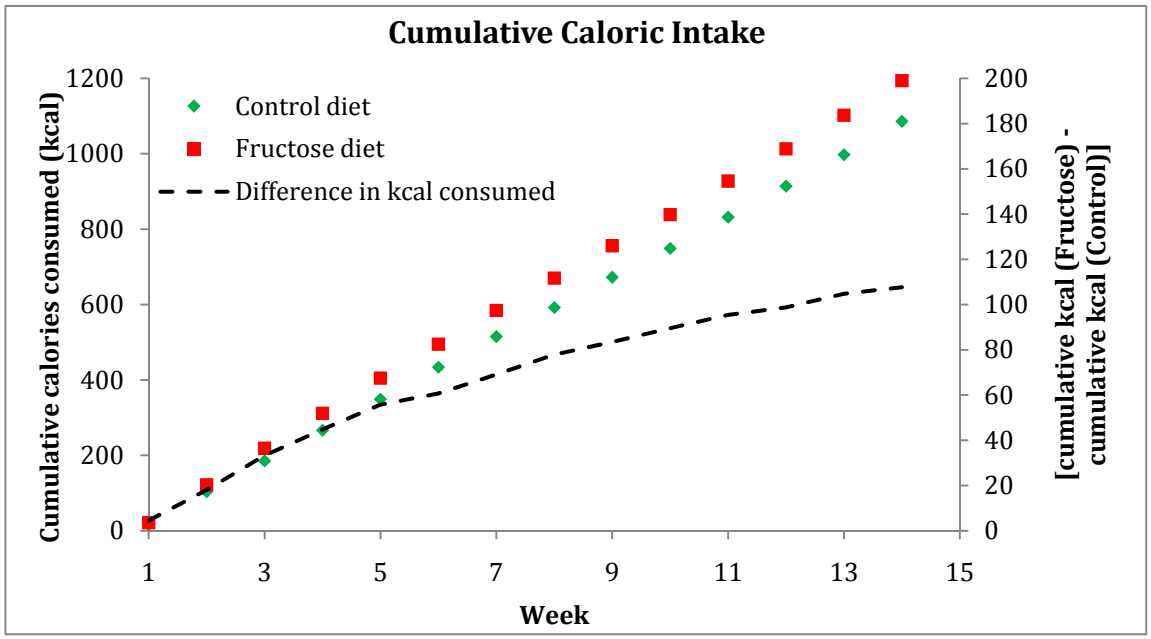

Figure 16- Over the course of the 14 weeks, fructose-fed mice (n=19) consumed more kcal than control-fed mice (n=19) both cumulatively, and on a week-by-week basis ($p<0.05$ for all weeks).

Body Weight

Over the course of the 14 weeks, body weight was measured 5 times weekly. At the beginning of the study, mice that were subsequently fed high fructose food (17.5 ± 0.4 g) did not significantly differ in body weight from mice that were subsequently given a fructose-free diet (17.6 ± 0.5 g). After 7 weeks, mice on the high fructose diet (23.1 ± 0.3 g) had significantly higher body weights than mice on the control diet (21.4 ± 0.3 g). However, after 14 weeks, mice on the high fructose diet (26.1 ± 0.4 g) did not have body weights that significantly differed from mice on the control diet (26.2 ± 0.5 g) (Figure 17).

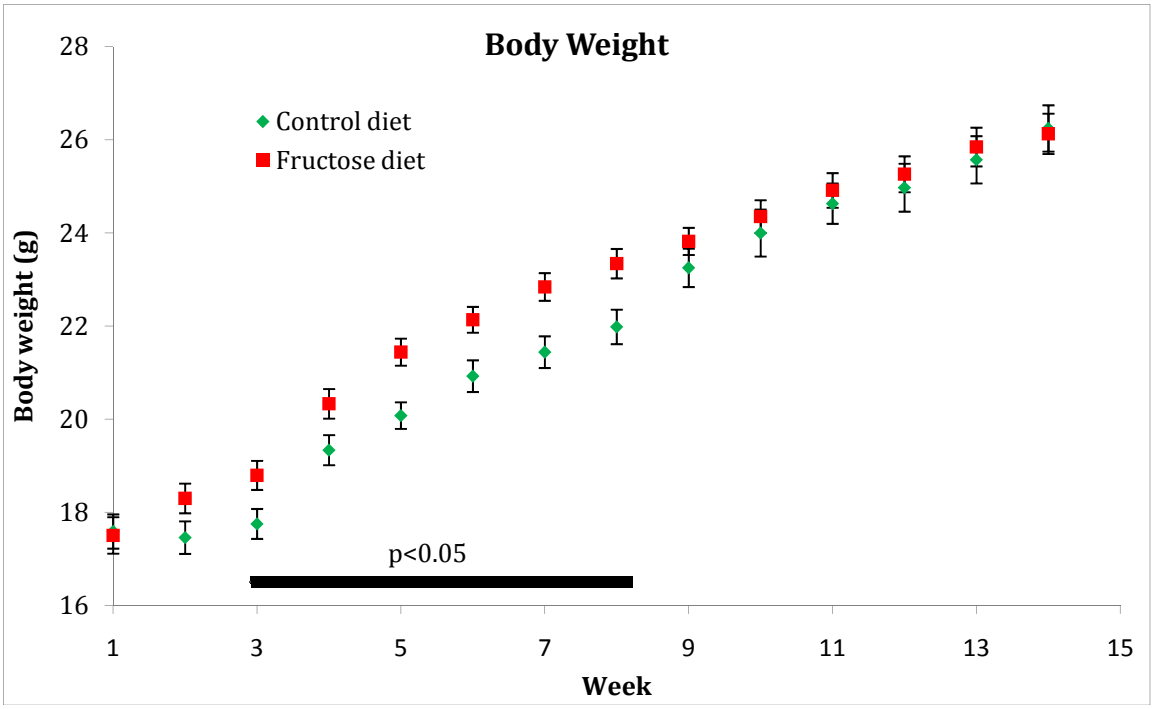

Figure 17- After 14 weeks on their respective diets, control-fed (n=19) and high-fructose-fed (n=19) mice weighed the same, although there appears to be developmental delay in the control-fed mice.

Metabolic Rate and Energy Balance

Energy expenditure was higher ($p<0.05$) for the high-fructose-fed mice than the control-fed mice in weeks 2, 4, and 9 (Figure 18). In week 2, high-fructose-fed mice (n=7) expended 14.3 ± 0.4 kcal/24 hours, whereas control-fed mice expended 12.8 ± 0.6 kcal/24 hours. In week 4, high-fructose-fed mice expended 8.2 ± 0.2 kcal/24 hours, whereas control-fed mice expended 5.9 ± 0.7 kcal/24 hours. In week 9, high-fructose-fed mice expended 10.6 ± 0.9 kcal/24 hours, whereas control-fed mice expended 8.5 ± 0.4 kcal/24 hours. In weeks 7, 11, and 14, there was no difference in metabolic rates.

Figure 19 is a graph of metabolic rate over 1 one-day period for high-fructose-fed and control-fed mice. Mice on the experimental diet had significantly higher metabolic rates than the control-fed mice for much of the 24-hour period.

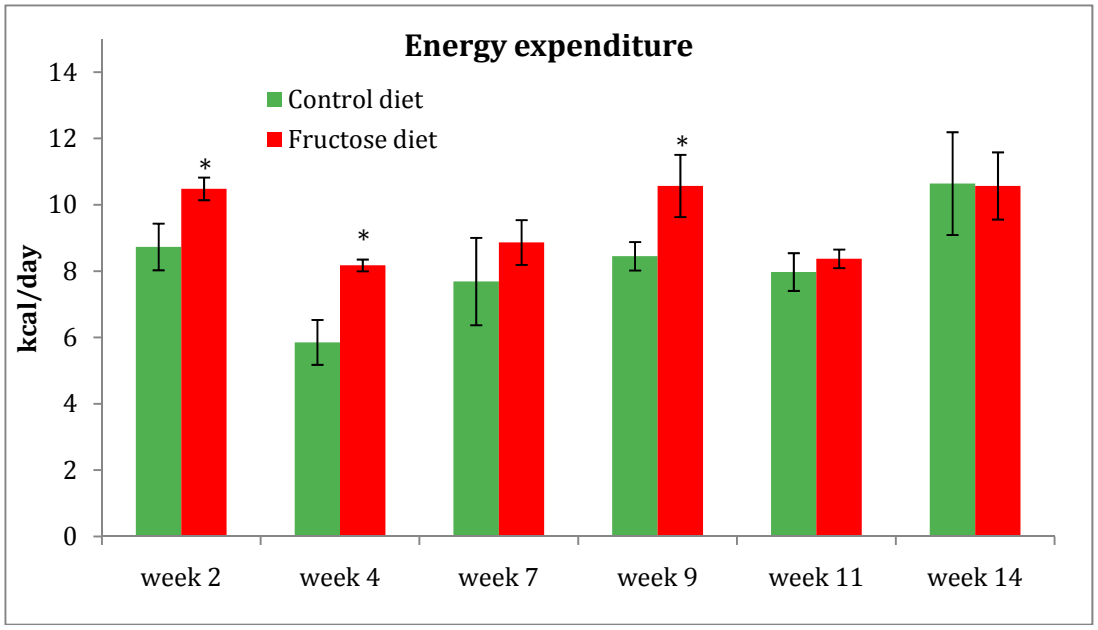

Figure 18- In weeks 2, 4, and 9, fructose-fed mice (n=7) had elevated metabolic rates compared to control-fed mice (n=7). This difference was not present in weeks 7, 11, and 14 (*- $p<0.05$ compared to control diet for indicated week).

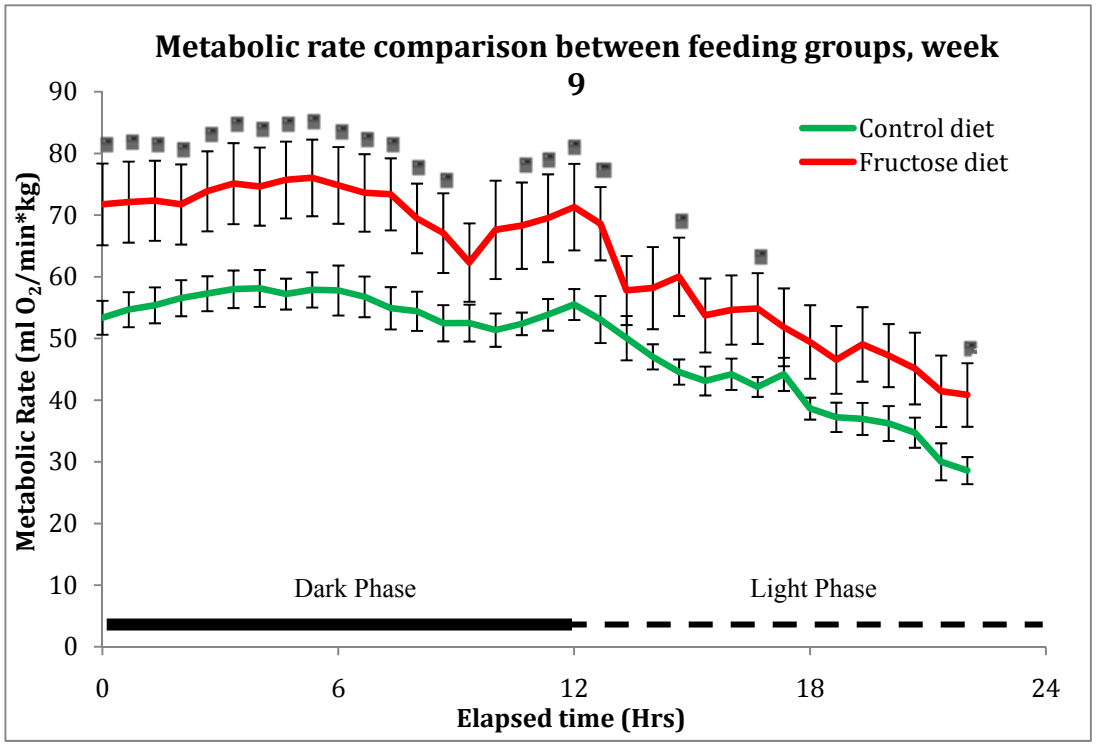

Figure 19- Representative comparison of metabolic rates between high-fructose-fed (n=7) and control-fed (n=7) mice, week 9. Each data point represents a condensed 40-minute average for all the mice in that feeding group (*- $p<0.05$ compared to control diet for indicated time point).

Elevated metabolic rate in weeks 2, 4, and 9 in high-fructose-fed mice was accompanied by higher caloric intake in fructose-fed mice during those weeks (Figure 20). Additionally, when the difference in metabolic rate between the feeding groups disappears, as in week 11, caloric intake is similar between the groups as well.

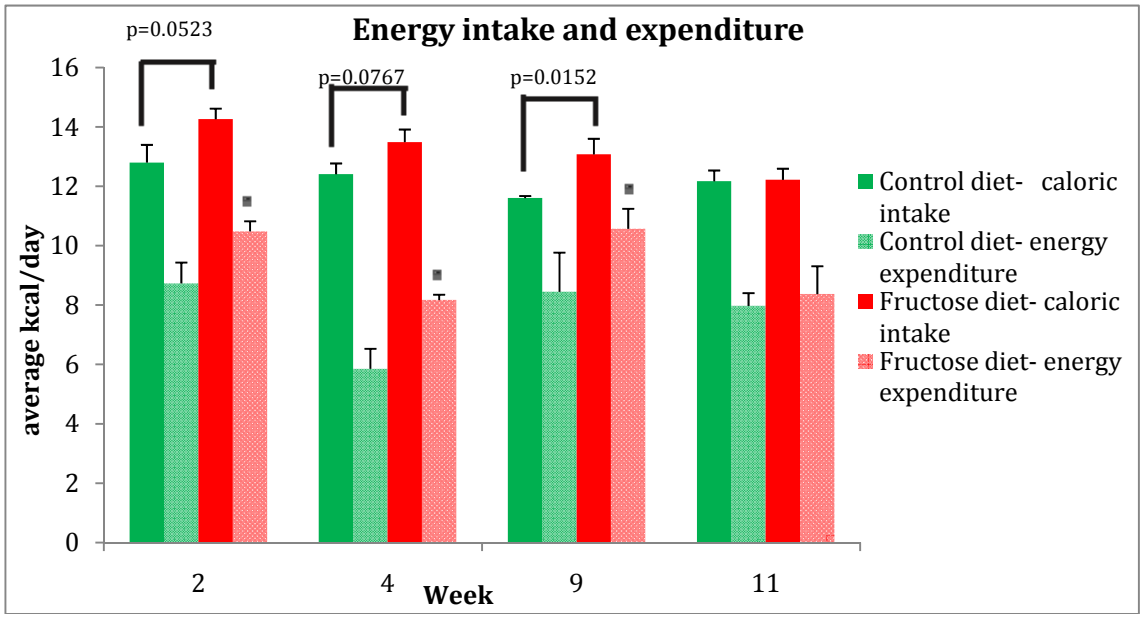

Figure 20- Elevated caloric intake in fructose-fed compared to control-fed mice in weeks 2, 4, and 9 is accompanied by elevated energy expenditure in fructose-fed compared to control fed mice (*-$p<0.05$), such that energy balance is similar between fructose-fed and control-fed mice. Similar caloric intake between the feeding groups in week 11 is accompanied by comparable energy expenditure.

Leptin Sensitivity

Caloric intake (Figure 21) was measured for the two days prior to injection with either vehicle or leptin, and a 24-hour average caloric intake was calculated for mice in each feeding group. Control-fed mice consumed 12.6 ± 0.3 kcal/day, and high-fructose-fed mice consumed 12.9 ± 0.2 kcal/day during the two days prior to injection. Leptin administration caused significant decreases in food intake compared to vehicle injection

after 6 hours for both control-fed (4.7 ± 0.6 kcal vs. 6.1 ± 0.2 kcal; $p<0.05$) and fructose-fed (5.5 ± 0.3 kcal vs. 6.8 ± 0.5 kcal; $p=0.050$) mice. After 24 hours, mice consuming the control diet still had a significant decrease in caloric intake in response to the leptin injection compared to vehicle injection (10.1 ± 0.9 kcal vs. 13.4 ± 0.4 kcal; $p<0.05$) but leptin administration to high-fructose-fed mice led only to a downward trend in caloric intake in the 24 hours post-injection compared to vehicle injection (12.9 ± 0.8 kcal vs. 15 ± 0.9 kcal; $p=0.053$). Caloric intake in the second 24-hour interval after leptin injection was not different from vehicle injection for either control-fed (11.4 ± 0.4 kcal vs. 11.4 ± 0.4 kcal) or high-fructose-fed (13.4 ± 0.7 kcal vs. 13.4 ± 0.7 kcal).

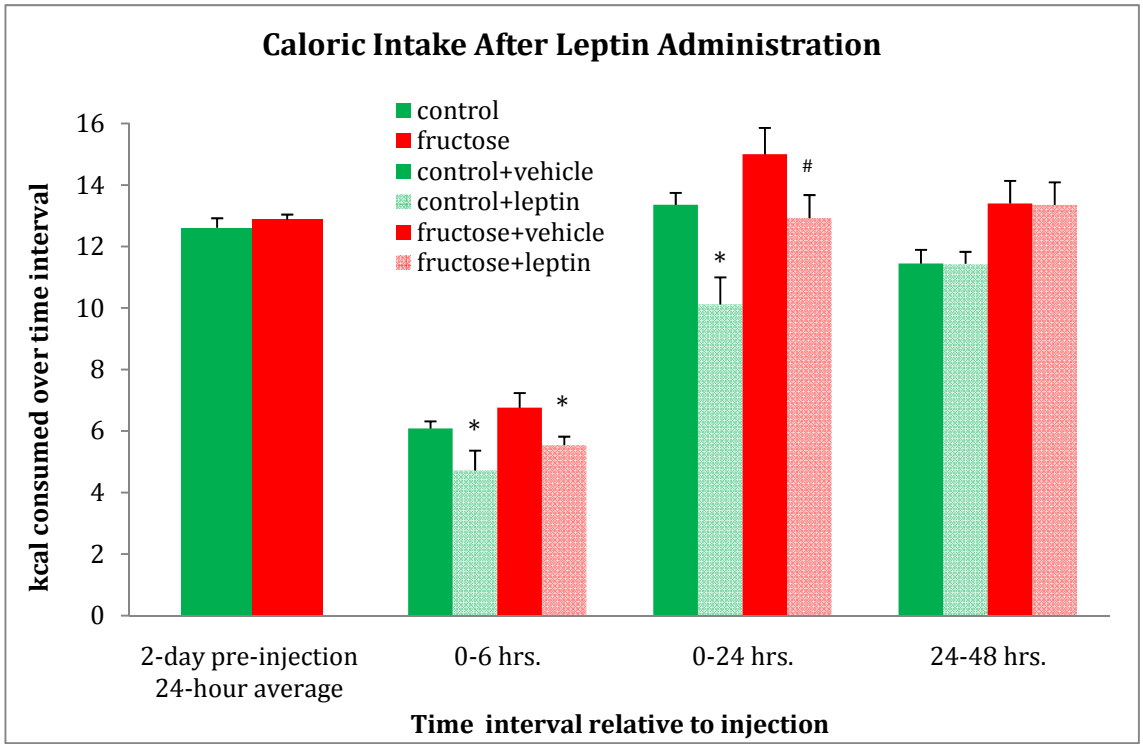

Figure 21- Leptin injection leads to decreased food intake in both control-fed and fructose-fed mice compared to injection of vehicle over 6 and 24 hours, and returns to normal after 24 hours (*-$p<0.05$; #-$p=0.053$). The first time point is a 24-hour average of the two days prior to the injection. Other time points are total caloric intake over indicated time interval after injection.

Body weights (Figure 22) were measured at the time of injection, and 6, 24, and 48 hours thereafter. Weights across the groups were not significantly different at any of the time points. At the time of injection, control-fed mice injected with vehicle weighed 26.3 ± 0.6 g, and those injected with leptin weighed 26.0 ± 0.7 g; high-fructose-fed mice injected with vehicle weighed 26.2 ± 0.7 g, and those injected with leptin weighed 26.1 ± 0.6 g. Six hours after the injections, control-fed mice injected with vehicle weighed 27.2 ± 0.5 g, and those injected with leptin weighed 26.3 ± 0.9 g; high-fructose-fed mice injected with vehicle weighed 27.3± 0.8 g, and those injected with leptin weighed 26.2 ± 0.7 g. Twenty four hours after the injections, control-fed mice injected with vehicle weighed 26.5 ± 0.6 g, and those injected with leptin weighed 25.9 ± 0.8 g; high-fructose-fed mice injected with vehicle weighed 26.7± 0.7 g, and those injected with leptin weighed 26.3 ± 0.6 g.

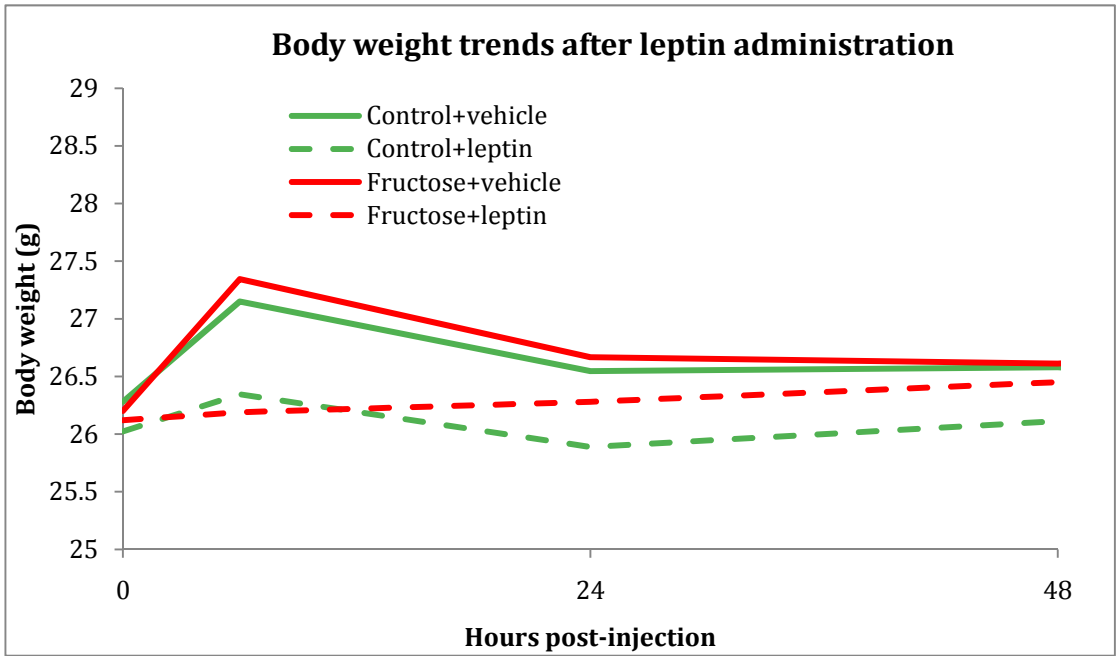

Figure 22- Leptin injection does not significantly alter body weight in either feeding group compared to vehicle. While leptin causes blunted body weight increases over the first 6 hours after injection, this trend is not significant.

Plasma Biomarkers

Mice from both feeding groups were randomly selected for either a 24-hour fast or continued feeding while metabolic rate was measured. After acquiring metabolic data, the mice were sacrificed 2 hours prior to the onset of the dark phase, and plasma was isolated. Table 2 shows the results of the assays performed on collected plasma. The only significant finding was that high-fructose-fed mice, when fasted, have elevated free fatty acid levels compared to both control-fed fasted mice and high-fructose-fed mice ($p<0.05$).

Table 2- Fed and fasting plasma glucose, triglyceride, and free fatty acid levels. Glucose and triglyceride levels are comparable for control- and high-fructose-fed mice, whether fed or fasted. Fasted high-fructose-fed mice had elevated fasting non-esterified fatty acid (NEFA) levels compared to control-fed mice (*-$p<0.05$ vs. high-fructose fasted condition).

		Control diet	High-fructose diet
[Glucose] (mg/dL)	Fasting	73.2 ± 1.3	67.0 ± 2.9
	Fed	81.4 ± 8.2	81.0 ± 5.4
[Triglycerides] (mg/dL)	Fasting	26.1 ± 3.6	28.8 ± 5.7
	Fed	23.6 ± 3.9	17.6 ± 2.7
[NEFA] (mmol/L)	Fasting	76.5 ± 13.1 *	137.8 ± 28.1
	Fed	43.0 ± 7.2	32.3 ± 6.0 *

DISCUSSION

Experiment 1

Caloric Intake and Body Weight

Over the course of the diet regimen, control-fed mice appeared to consume more on a daily basis than mice fed a high-fructose diet (Figure 10). While it is possible that the control-fed mice actually ate more, it is also likely that difficulties in accurately measuring food consumption led to a falsely elevated caloric intake for the control-fed mice compared to mice fed a high-fructose diet.

The control diet became desiccated at a faster rate than the high-fructose diet, leading to frequent crumbling of the control diet. Also, because the control diet was drier, it was more easily removed from the food dishes by the mice and lost in the bedding of the cages. While large chunks of control food could usually be recovered from the bedding, on days when cages were changed, these large pieces of food were discarded before they could be counted, further contributing to the potential inflation of food consumption data for control-fed mice.

As control food consumption was likely overestimated in experiment 1, I cannot draw strong conclusions about which feeding group had mice that consumed more kcal. While I hypothesized that high-fructose-fed mice would consume more kcal than control-fed mice, I cannot reject the null hypothesis from the observations of the 3-week feeding study.

Mice consuming high-fructose food did not have significantly higher body weights than control-fed mice, though the data show a trend of increase over time (Figure 11). The fact that body weights were comparable implies that energy balance between

the two feeding groups was similar over the course of the study. If Figure 10 shows correct caloric intake and not merely food removed from, but not necessarily eaten, dishes, then the control-fed mice would need to expend more energy to maintain an energy balance similar to that of the high-fructose-fed mice.

An observation pertinent to the apparent lower body weights in control-fed mice relates to the food itself. Because the control diets tended to be drier and crumble much more easily, there were frequently small crumbs of up to 0.5 g remaining in the dishes. If the crumbs were not palatable, then the mice could have been calorically restricted while my method of measuring caloric intake would show that there was food they had not been eating.

This was supported by the changes in weight after caloric intake and body weight measurements halted but mice were continued on their diets, and mice were fed ad libitum and so were receiving much more food on a daily basis than previously. Under these conditions, the body weights of the control-fed mice increased (data not reported). Therefore, if experimental conditions led the control-fed mice to be unintentionally calorically restricted, their body weights would be lower than if they had been fed ad libitum. Control-fed mice would therefore have an artifactual negative shift in energy balance. Despite the elevated observed caloric intake in control-fed mice, there are several explanations as to why the body weights did not differ, without calling on any metabolic data, which was not collected during the feeding period of Experiment 1.

Leptin Sensitivity

IP leptin administration did not decrease caloric intake significantly compared to saline administration in either the control-fed mice or the high-fructose-fed mice (Figure 12). In normal, leptin sensitive mice, exogenous leptin administered peripherally decreases energy intake and increases energy expenditure (Halaas, Gajiwala et al. 1995; Halaas, Boozer et al. 1997). While there was a downward trend in both fructose-fed and control-fed mice injected with leptin compared to saline, the decreases after 6 and 24 hours were not significant.

Small sample size may have contributed to the smaller than expected response to leptin, as each injection group had 5-6 mice. More likely, however, the improper reconstitution of the leptin prior to injection led to no observable physiological response. Leptin was reconstituted in saline solution, although ProSpec Bio's instructions called for reconstitution in 18MΩ-cm H_20 at a concentration far below what was necessary for injection. The leptin failed to go into solution in the saline and so the actual injected dose was likely far below 5 mg/kg.

Additional support for the interpretation that leptin failed to produce anorexigenic effects because of methodological issues comes from control injections on C57Bl/6 mice. Because these mice, which had been calorically restricted for days before injection, did not respond differently to saline versus leptin injections, I concluded that the leptin solution had been improperly prepared and could not inhibit appetite and stimulate satiety.

Changes in body weight were consistent across all diet and injection combinations after administration, further supporting the interpretation that leptin failed because of

methodological issues (Figures 13 and 14). All groups experienced an increase in body weight over the first 6 hours after leptin administration, which should not have occurred with the expected decreased caloric intake associated with leptin administration. Because the leptin was administered right before the start of the dark phase, the mice should normally eat at the beginning of their active period.

It was necessary to compare percent changes in body weight because mice were not weight-matched when assigned to injection groups, and so the initial body weight of control-fed mice injected with leptin was lower than the other three groups' body weights. Regardless of injection or feeding group, body weight increased 3%. I hypothesized that high-fructose-feeding would decrease peripheral sensitivity, but the data support rather than reject the null hypothesis.

Fasted Metabolic Rate

When fasted at 18°C, control-fed (n=2) and high-fructose-fed (n=3) mice had similar metabolic rates, and mice in neither experimental group entered torpor. Likely contributing to the lack of entry into torpor was the high body weight of the mice used in this experiment. The similarity in metabolic rates indicated similar energy expenditure, which, in conjunction with elevated caloric intake in control-fed mice, should create a larger positive shift in energy balance than in high-fructose-fed mice, and lead to greater weight gain in the control group. As body weights were similar, I conclude the overestimation of caloric intake in control-fed mice, as this is the measurement most susceptible to error based on the design of Experiment 1.

Conclusion for Experiment 1

A 3-week high-fructose diet does not induce leptin resistance in Hsd:NSA (CF-1) mice. Because of the problems inherent in the design of Experiment 1, I decided to feed smaller, C57Bl/6 mice a pre-made diet for longer in Experiment 2.

Experiment 2

Caloric Intake and Body Weight

Over the course of 14 weeks, high-fructose-fed mice had higher caloric intake, each week and cumulatively (Figure 16). The constantly increasing difference between high-fructose cumulative kcal consumed and control kcal consumed indicates a chronic trend of elevated food intake in high-fructose-fed mice compared to control-fed mice illustrate that every week, the high-fructose-fed mice were eating more, and the difference in cumulative kcal consumed was not punctuated, but continuous. The use of pelleted food from Teklad eliminated the problem from Experiment 1 with substantial amounts of either diet being unintentionally discarded, and so the observed caloric intake can be taken as a much more accurate estimation of the actual caloric intake of the mice.

Body weight was the same for high-fructose-fed and control-fed mice at the beginning of the feeding study and after 14 weeks on the diet (Figure 17), indicating similar energy balance between the feeding groups. High-fructose-fed mice were significantly heavier than control-fed mice between weeks 3 and 8, likely due to a slower acclimation of mice to the control diet than to the high-fructose diet. The increased slope of the line in Figure 16 in earlier weeks corresponds to cumulative kcals consumed diverging more rapidly, i.e. a larger daily difference between high-fructose and control kcal consumed early in the feeding study than later. The increased sweetness of the high-

fructose diet may be responsible for this phenomenon, which is supported by the observation that an initial increase in body weight, once switched to either experimental or control diet, occurs weeks later in control-fed mice (Figure 17).

Because similar body weights were recorded at the end of the study for the two feeding groups despite 14 weeks of chronically increased food intake in high-fructose fed mice, there must be a force acting to stabilize the energy balance of the high-fructose-fed mice by shifting energy balance in the negative direction. Possibilities for increasing energy expenditure include increased thermogenesis, increased locomotor activity, or increased basal metabolism in high-fructose-fed mice (Figure 1). All of these would be observable as an increased metabolic rate compared to control-fed mice.

Metabolism and Energy Balance

For the first 9 weeks of the study, though not week 7, metabolic rate was higher in high-fructose-fed mice than in control-fed mice (Figure 18). For weeks in which energy expenditure was elevated, high-fructose-fed mice had higher metabolic rates in both the dark and the light phase (Figure 19). This elevation was accompanied by elevated caloric intake in the high-fructose-fed compared to control-fed mice (Figure 20). The elevated metabolic rate of mice consuming a high-fructose diet compared to a control diet, a negative energy balance shift, coupled with increased caloric intake of the mice on the high-fructose diet, a positive shift in energy balance, helps explain the similar body weights between the feeding groups after 14 weeks.

There are two possibilities that might explain the maintenance of energy balance in the mice on the high-fructose diet. The first is behaviorally driven; the high-fructose diet is considerably sweeter than the control diet, so mice may consume more of it on a

daily basis. Eating more leads to increased energy expenditure by the simple fact that digestion increases with food intake, and the digestion of food contributes significantly to metabolic rate (McArdle 1986). Thus, in this model, the mice eat more high-fructose food because it is sweeter, and eating more increases metabolic rate.

The second potential model is metabolically driven. If the fructose in the high-fructose diet increases metabolic rate somehow, then the mice would eat more to compensate for the initial negative shift in energy balance. A possible mechanism for such a fructose-driven increase in metabolic rate could be mediation by the generation of peroxynitrite ($ONOO^-$) from superoxide (O_2^-) and nitric oxide (NO). Consumption of fructose elevates myocardial O_2^-, which combines with NO to form $ONOO^-$ (Mellor, Ritchie et al. ; Koppenol, Moreno et al. 1992). As NO inhibits cellular respiration, and fructose-induced increase in cellular superoxide concentration driving the production of peroxynitrite would decrease the cellular concentration of nitric oxide, we have a possible mechanism by which fructose may increase cellular oxygen consumption.

Regardless of potential mechanism, I am unable to reject the null hypothesis, and must therefore conclude that the increased food intake and metabolic rate of mice fed the high-fructose diet maintained an energy balance comparable to that of the control-fed mice based on similar body weights after 14 weeks on respective diets.

These findings contrast with a recent study of the effects of HFCS on energy balance and susceptibility to obesity in rats (Bocarsly, Powell et al.). In my study, I used fructose in the diet, whereas Bocarsly et al. administered either HFCS, sucrose, or no sweetener dissolved in water and given free access to normal lab chow. Bocarsly et al. found that HFCS supplementation of a normal lab diet caused obesity, characterized by

increased body weight, increased abdominal fat, and elevated triglycerides, compared to rats fed chow supplemented with either sucrose or unsweetened water.

Leptin Sensitivity

After 14 weeks of high-fructose diet consumption, mice had a similar hypophagic response to leptin administration as did mice fed the control diet (Figure 21). Normal, leptin sensitive mice decrease food intake and lose body weight after administration of exogenous leptin. I hypothesized that the control-fed mice would remain leptin sensitivie, but that high-fructose feeding would attenuate peripheral leptin sensitivity. Because there was no difference in caloric intake after injection between the two feeding groups, and both groups decreased their caloric intakes, I must conclude that high-fructose-feeding over 14 weeks did not induce leptin resistance in the C57Bl/6 mice.

Shapiro et al. (2008) observed elevated circulating triglyceride levels with fructose-feeding induced leptin resistance, and speculated that high circulating triglycerides may impair permeability of leptin across the BBB, so I hypothesized that fructose-feeding in mice would elevate triglycerides, a potential mechanism of decreasing peripheral leptin sensitivity. However, triglyceride levels did not differ between high-fructose-fed and control-fed mice (table 2). Because a 14-week regimen of a high-fructose diet neither induced leptin resistance nor elevated circulating triglycerides, the earlier hypothesis that elevated triglycerides were directly responsible for leptin resistance through impairment of leptin permeability across the BBB is still feasible. If leptin resistance had been present without elevated triglycerides, the role of triglycerides would have to be disregarded, as it would if elevated triglycerides had been present in leptin sensitive high-fructose-fed mice.

Despite the fact that chronic high-fructose feeding did not induce leptin resistance in C57Bl/6 mice in my experiment, investigations into mechanisms of developing leptin resistance and the effects of dietary fructose are important in the fields of obesity and nutrition. When developed, leptin resistance leads to a positive shift in energy balance and, consequently, obesity. I found that dietary fructose induces neither leptin resistance nor a change in energy balance in mice, but fructose may have other negative physiological effects not tested in this thesis. An important further study would be the metabolic and physiological difference between the monosaccharide fructose, the disaccharide sucrose, and HFCS in normal dietary concentrations, to address whether a rise in obesity is attributable to increased fructose consumption, increased HFCS consumption, or simply too much sugar.

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