THE UNIVERSITY OF CHICAGO

FRUCTOSE METABOLISM IN FVB/N MICE:
DIFFERENTIAL EFFECTS OF LONG- AND SHORT-TERM FEEDING

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List of Abbreviations

SES	socioeconomic status
SRF	sugar research foundation
CHD	coronary heart disease
HFCS	high-fructose corn syrup
KHK	ketohexokinase
AldoB	aldolase b
NAFLD	nonalcoholic fatty liver disease
GTT	glucose tolerance test
IACUC	institutional animal care and use committee
DEXA	dual-energy x-ray absorptiometry
RER	respiratory exchange ratio
CV	caloric value
WAT	white adipose tissue
BMD	bone mineral density
HDL	high-density lipoprotein
DHAP	dihydroxyacetone phosphate
TKFC	triokinase / fmn cyclase
PKL	pyruvate kinase
HPA	hypothalamic – pituitary – adrenal
11-DHC	11-dehydrocorticosterone
11β-HSD1	11 Beta-hydroxysteroid dehydrogenase 1
FGF-21	fibroblast growth factor – 21
PPARγ	peroxisome proliferator-activated receptor gamma
ACC	acetyl-CoA carboxylase
ACLY	ATP citrate lyase
ACSS2	acyl-CoA synthetase short chain family member 2
FASN	fatty acid synthase

Acknowledgments

The past five years have been complex times – full of excitement and success, but also some disappointment and even failure. I think that probably outlines how most people view the scientific method though, and I am both proud of myself for the achievement and excited for what the future has in store. It is clear that none of what I accomplished could have been possible without the mentorship, assistance, and inspiration of many people.

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Abstract

Obesity rates have been climbing steadily for the past hundred years, and in conjunction so have the rates of many metabolic diseases. The reasons for this are both complex and multifaceted. Homecooked meals have been replaced by many highly processed foods, while lifestyles have become more sedentary. Throughout this time, as obesity rates have grown, the consumption of fructose has increased in parallel. In addition to the correlation between fructose consumption and obesity, fructose has been linked to the development of multiple metabolic diseases, including hypertension, NAFLD, diabetes, and cancer. While great strides have been made to begin to understand these associations, more work is required to mechanistically link fructose metabolism to the development of metabolic disease. More specifically, evidence is lacking for how fructose catabolism is altered after the continued ingestion of a high-fructose diet for long periods of time, and how these specific changes to metabolic function are hypothesized to drive the development of metabolic diseases that continue to plague society. In the following chapters we strove to answer some of these questions by investigating the effects of long-term high-fructose feeding in FVB/N mice. Overall, these mice were resistant to the development of fructose-driven obesity and glucose intolerance, while still showing evidence of metabolic disturbance in peripheral tissues. Furthermore, while short-term high-fructose feeding in these mice led to an increase in fructolytic and adipogenic genes, as expected, long-term high-fructose feeding no longer caused these same metabolic genes to be elevated. These data provide evidence for a fructolytic pathway that cannot be maintained after excessive and prolonged high-fructose feeding.

Chapter I

The obesity epidemic and fructose metabolism: an overview

A. Introduction

In the past hundred years many great achievements have improved the human condition. Industrialization and globalization have led to multiple changes throughout society which have increased lifespan, and to a lesser extent healthspan. Access to education, improving literacy rates, and urbanization have caused a significant decline in severe poverty. Improvements to engineering and technology have made many aspects of life safer and less physically demanding. Medical innovations have completely changed the causes of mortality. Many accidents, like fractures, burns, and infections, are much more treatable and only rarely lead to death. For centuries, communicable diseases were one of the leading causes of death around the globe; however, the creation of vaccines and antimicrobial therapies have rendered them treatable and eradicated many of these diseases throughout the developed world.

Due to these innovations, and others, life expectancy has increased by over twenty years since 1900. With this shift towards an aging population and the aforementioned improvements to healthcare, causes of death have also changed. Cardiovascular disease and cancer are now the leading causes of death throughout the world. While this is in part due to agding populations, rates of obesity and metabolic diseases – leading risk factors for both cardiovascular disease and cancer – have drastically increased in recent years.

One consequence of the industrialization and globalization that continues around the world is that people live more sedentary lives, while also have greater access to food [1]. Since obesity occurs as people consume more calories than the demands of their body, the coexistence of greater food consumption and a sedentary lifestyle has caused a dramatic spike in the prevalence of obesity. Moreover, the standard diet has also changed. Home-cooked meals have been replaced by calorically dense foods, which are high in both fat and sugar content but may lack other desired nutrients [2].

One sometimes overlooked dietary component that can be influential in the development metabolic syndrome is fructose. Although found naturally in fruits and honey, fructose has been used increasingly as an artificial sweetener due to its inexpensive production and sweet taste. Due to this, fructose consumption has increased dramatically over the past forty years, paralleling the rise in obesity and many of its comorbidities [3]. While both fructose and glucose are six-carbon sugar molecules, differences to their functional groups make the absorption and metabolism of these sugars completely different. For reasons that are only partially understood, significant consumption of fructose has been linked to increased rates of obesity, metabolic syndrome, and many corresponding comorbidities.

To ensure that both lifespan and healthspan continue to increase over the next hundred years steps must be taken to both understand and address the ongoing obesity pandemic, and how fructose consumption may be partly to blame for the increased prevalence of many metabolic diseases.

B. The obesity epidemic and metabolic disease

Obesity is a complex yet largely preventable disease, with overwhelmingly negative health consequences. Obesity can be defined simply as excessive fat accumulation that occurs over time when energy intake is greater than energy expenditure. Throughout much of the world calorically dense food is readily available, occupational activities have changed, and regular exercise is uncommon, which have caused a significant increase in obesity rates worldwide. Over the past forty years global obesity has tripled and in certain populations this increase is far more pronounced. According to the World Health Organization, in 2016 39% of adults worldwide were overweight and 13% were obese. In contrast, in the United States of America 39.8% of adults and 18.5% of children are obese according to data from the National Health and Nutrition Examination Survey in 2016 [4].

An individual's risk for obesity is multifaceted and is influenced by their genetic make-up, socioeconomic status (SES), activity level, sleep habits, and diet [5]. For this reason, it is difficult to attribute a specific cause for the dramatic increase in obesity observed in the past four decades; however, the impact has not gone unnoticed. Medical costs attributed to obesity were approximately $40 Billion in 2006, but had increased to $150 Billion in 2014, and is estimated to approach $210 Billion in the near future [6]. Much of this substantial cost is attributed to the treatment and management of comorbidities associated with obesity, including diabetes and cancer.

During times of caloric surplus, adiposity increases as a way to store energy that can be utilized during periods of caloric deficit. The ability to efficiently store energy for

future fasts has been extremely advantageous throughout evolutionary history. Most simply, the body can extract energy from food by breaking chemical bonds in the carbohydrates, fats, and proteins that make up much of the diet. For this reason, prolonged consumption of high-caloric diets and minimal exercise is significantly associated with an increase in adiposity.

Due to fatty acid storage in adipocytes, dietary fat has been long regarded as a primary cause of obesity [7]. Excessive consumption of dietary fat is strongly associated with development of diet-induced obesity, as well as induced metabolic shifts in tissues throughout the body [8]. While the consumption of dietary fat has been associated with the development of obesity, metabolic syndrome and cardiovascular disease for many years, the chronic overconsumption of sugar has been relatively understudied until recently. This is partly due to how the sugar industry and the Sugar Research Foundation (SRF) concealed scientific findings which strongly linked the consumption of sugar to the development of coronary heart disease (CHD), cancer, and obesity [9, 10]. Instead, the consumption of fat was considered the sole culprit for the development of these diseases for many years. Due to this obfuscation of the facts for monetary gain, there is still much that is unknown about sugar consumption and the subsequent development of metabolic diseases.

In addition to the digestion of fatty acids, simple sugars are also readily absorbed and metabolized. Glucose, fructose, and galactose are the three monosaccharides that can pass through enterocytes to enter the hepatic portal vein and be metabolized by the liver. As glucose is the main sugar substrate utilized in the human body, much of the fructose and galactose absorbed is converted into glucose or other carbon molecules

used readily in cellular metabolism. A molecule of glucose can quickly be used by the cell to create energy via the glycolytic pathway and subsequent oxidative phosphorylation. These metabolic pathways catabolize glucose into carbon dioxide and water to efficiently create energy intermediates like ATP and the electron carrier NADH. The ability to rapidly create usable energy in the cell during times of increased demand essential for any living organism. However, when the amount to glucose available surpasses the needs of the organism, it is vital to store the chemical energy obtained from glucose catabolism, so it can be utilized when carbohydrates are scarce.

Sugar consumption, obesity, and Type II diabetes mellitus are tightly linked. Being overweight or obese raises one's chance of developing diabetes three to seven times, respectively. Furthermore, more than 90% of people with diabetes are overweight or obese [11]. The human body does an exceptional job of maintaining blood glucose levels within physiological limits – during both fed and fasted states - as deviation past the homeostatic set point in either direction can lead to numerous medical complications.

Throughout fasting periods, blood glucose is maintained by breaking down energy-dense molecules stored in the liver, adipocytes and skeletal muscle – triglyceride and glycogen. This catabolic state is mainly under the control of glucagon, which is produced by the alpha cells of the pancreas to increase glucose and fatty acid levels in the blood. Conversely, after a meal, when blood glucose levels are high, the beta cells of the pancreas release the hormone insulin, which acts to lower blood glucose levels and induce lipogenesis. With chronic over-secretion of insulin, cells throughout the body can become resistant to the actions of the hormone. As this occurs overtime, the beta cells of the pancreas will compensate by secreting increasing amounts of insulin. Eventually, with

continued and chronically increased secretory demand, the beta cells will fail to produce of enough insulin to maintain normal blood glucose levels – this is the definition Type II diabetes mellitus [12]. As such, elevated blood glucose levels observed in patients with diabetes are treated with exogenous insulin. With the chronic overconsumption of calories causing the overstimulation of insulin secretion and increased rates of lipogenesis, it is easy to predict the rise of diabetes and obesity in the same patient populations. However, the metabolic changes that accompany the progression of obesity are associated with other diseases as well. The cluster of metabolic conditions that occur together which increase one's risk for heart disease, stroke, diabetes, and other health conditions is known as metabolic syndrome. The development of metabolic syndrome is associated with a poor diet high in fat, sugar, or even specifically fructose [13].

C. Metabolism of fructose

Glucose, along with fructose and galactose, are the three monosaccharides readily absorbed and metabolized as part of a standard diet. While glucose is catabolized through the highly controlled glycolytic process, both fructose and galactose begin to be broken down via distinct metabolic pathways – to be inserted at a lower point in the glycolytic pathway. Almost all dietary galactose is consumed as part of the disaccharide lactose, while fructose is found naturally in fruits and vegetables. In these small quantities, fructose and galactose are easily metabolized; however, large quantities of dietary fructose, mainly from the ingestion of processed foods and soft drinks, are now

known to cause distinct metabolic changes associated with numerous negative health outcomes [14].

While both glucose and fructose are six carbon sugar molecules, glucose is an aldohexose and fructose is a ketohexose. The differences in their structure cause fructose to be perceived as much sweeter than that of glucose alone. Because it is sweeter than glucose in equal concentrations, fructose has been used increasingly as an added sweetener. To be used more efficiently as a sweetener, high-fructose corn syrup (HFCS) began to be used in many industrial situations. HFCS is made by breaking down polymers of cornstarch into glucose molecules and converting a portion of those into fructose via a glucose isomerase based enzymatic reaction. Being inexpensive to produce, easily stored, and extremely sweet, HFCS has been used extensively as a sweetener of processed foods. Starting with the introduction of HFCS into the western diet in the 1970's, the average person now consumes more than 80 grams of HFCS per day, having a drastic impact on the prevalence of obesity and metabolic syndrome [3]. Furthermore, most of the downstream metabolites of the unregulated fructolysis leads toward either lipogenesis or gluconeogenesis – enhancing energy storage via glycogen and adipose tissue – which is expensive for the liver to maintain.

Due to fundamental differences in structure when compared to glucose, fructose is metabolized uniquely, utilizing different absorption and catabolic pathways. Once ingested, much of dietary fructose is metabolized in the small intestine – into glucose, acetate, glycerate, and lactate [15]. This is hypothesized to protect the liver from the harmful effects of fructose metabolism, but can also provide the acetate to feed the lipogenic pools of acetyl-CoA [16, 17]. Despite the small intestines attempt to buffer the

liver's exposure, some fructose will reach the liver, especially in states of high consumption. Fructose that is absorbed is transported across the membrane of intestinal enterocytes through the GLUT5 and GLUT2 transporters (encoded by the Slc2A5 and Slc2A2 genes, respectively) into the hepatic portal vein, where it is transported to the liver [18, 19]. In the liver, fructose, unlike glucose, is rapidly phosphorylated by ketohexokinase (KHK), bypassing hexokinase and phosphofructokinase – the rate limiting steps of glycolysis [20]. Furthermore, the ATP utilized by KHK can lead to a depleted pool of ATP when the following metabolic steps cannot keep up with the phosphorylation of fructose [21, 22]. The growing reservoir of fructose-1-phosphate is subsequently cleaved into DHAP and glyceraldehyde by aldolase B (AldoB), the rate-limiting step of fructolysis. These triose phosphates are further metabolized to pyruvate and glycerol-3-phosphate to create lactate and acetyl-CoA which will be utilized to create lipid. As fructolysis is essentially unregulated, large quantities of fructose are quickly metabolized into lactate, glucose, and fatty acid in the liver, regardless of energy balance [23]. Indeed, increased serum levels of lactate and triglyceride are observed after the ingestion of fructose [24].

Due to the unregulated catabolism of fructose, and subsequent fatty acid production, dietary fructose has been identified as a positive regulator in the development of many diseases, including nonalcoholic fatty liver disease (NAFLD), coronary heart disease (CHD), hypertension, obesity, and diabetes [25]. Interestingly, due to how fructose, unlike glucose, cannot directly stimulate insulin release, some physicians originally hypothesized that fructose consumption would have positive benefits for diabetic patients. Unfortunately, while fructose does not directly stimulate insulin release, endogenous glucose production is known to be upregulated. In addition to regulating

lipogenesis, carbohydrate response element binding protein (ChREBP) is also a potent activator of glucose-6-phosphatase – the terminal enzyme of gluconeogenesis [26]. Through these mechanisms, high-fructose feeding over time can lead to hepatic insulin resistance [27, 28]. The unregulated catabolism of fructose can strongly influence hepatic and systemic metabolism of glucose and lipid, and over time this can lead to the development of multiple diseases.

D. Summary

Due to the unique structure of fructose, it must be converted into glucose or other glycolytic intermediates to be utilized by most tissues in the body. Furthermore, the process of hepatic fructolysis is unregulated, as it bypasses many of the rate limiting steps of glycolysis before being reinserted as various carbon intermediates further down the glycolytic pathway. Although the negative health consequences of high-fructose feeding have begun to be understood, the complete understanding of how fructose-specific metabolism leads to these negative outcomes is yet to be fully developed.

In the following chapters we interrogate the health outcomes of long-term high-fructose feeding while investigating the hepatic response to fructose over that time. Interestingly, the majority of studies investigating the specific metabolism of fructose are short-term studies which study the effects of a fructose challenge. These studies are useful, as the prevalence and consumption of fructose-sweetened beverages has greatly increased in recent years. Furthermore, the entirety of fructose tracer studies have been completed in this manner, where a challenge of fructose is given and the consumption of

those fructose molecule are traced over a period of hours. Again, while there is great need for this type of investigation, it is imperative that long-term investigations of fructose metabolism are conducted.

As it is well-described in the development of many metabolic diseases, the body is capable of handling a single insult, but repeated insults for weeks, months, or years can significantly alter metabolism and be the root cause of disease progression. For this reason, long-term fructose studies are required to understand how repeated consumption of fructose may alter its described fructolytic pathway, and how this may lead to the progression of disease. As prospective studies in humans, especially one's focused on the metabolism of a potentially harmful substrate, are challenging, throughout the following chapters the effects of long-term fructose metabolism will be studied in FVB/N mice.

Due to the high energy demands of continued fructolysis, we hypothesize that the liver will adapt to the extended fructose challenge and that these adaptions may drive some of the long-term negative health outcomes associated with high-fructose feeding. As the rates of obesity and fructose consumption continue to climb in parallel, it is imperative to gain a more complete understanding of how fructose alters metabolism – not only immediately after consumption, but over time – and leads to the progression of multiple diseases.

Chapter II

Health outcomes of high-fructose feeding in FVB/N mice

A. Introduction

As outlined in chapter 1C, fructose is absorbed and initially catabolized by distinct mechanism when compared to glucose. When fructose reaches the liver after excessive consumption, it is rapidly metabolized – bypassing hexokinase and the other initial rate-limiting steps of glycolysis – to create triose phosphates that enter lower into the glycolytic pathway. These hepatic metabolic intermediates produced from excessive fructose consumption are shunted toward the creation of glucose, lactate, and fatty acids. During periods of exercise, these substrates can be utilized by muscles and other tissues for energy production; however, in sedentary individuals, the excess triose phosphates are mainly utilized for hepatic de novo lipogenesis [29]. Furthermore, the unregulated nature of fructose metabolism is known to cause depletion of hepatic ATP and increased nucleotide turnover [30]. Together, it is not surprising the a high-fructose diet is associated with the onset of many diseases, including hypertension, CHD, NAFLD, and cancer [31-35]. While the deleterious effects of high-fructose feeding are beginning to be comprehended, much work is required to fully understand the mechanisms of onset and progression for these diseases.

Around the world, the consumption of fructose continues to increase, but a well-controlled prospective study of increased fructose consumption remains challenging in human populations due to ethical concerns. For this reason, many animal models have

been used for a more thorough analysis of how metabolism is altered after high-fructose feeding. Over the past decades, the use of C57BL/6 mouse strains have been utilized in the overwhelming majority of metabolic studies. While the abundance of experimentation in a single mouse background, and subsequent knowledge gained, allows greater comparability between studies, no one mouse can perfectly capitulate human metabolism. For example, the C57BL/6 background is highly susceptible to diet-induced obesity which allows for necessary testing on how obesity is developed; however, the presence of obesity emits specific metabolic changes regardless of other factors, like diet [36, 37]. For this reason and others, it is imperative to study multiple animal models when studying various metabolic pathways and how they are effect by specific diets.

Throughout these studies, FVB/N mice are utilized to interrogate the metabolic consequences of high-fructose feeding. These investigations are some of only a few studies that have explored the effects long-term high-fructose feeding or the effects of fructose consumption on FVB/N mice. FVB/N mice are known to be resistant to the development of diet-induced obesity, which allows for a direct and thorough investigation into the metabolic consequences of high-fructose feeding without the confounding effects of obesity [38].

The goal of this study is to characterize the metabolic response of FVB/N mice to long-term high-fructose feeding. This novel study will provide important health outcome data in these mice, which will be used to better understand how metabolism is altered in the following chapters. Using an array of techniques, including metabolic cage testing, adipocyte histology, and glucose tolerance tests (GTTs), FVB/N mice were found to have

an increase in adipocyte size without significant changes in weight gain or peripheral glucose metabolism after twelve weeks of high-fructose feeding after weaning.

B. Materials and methods

Animal care: This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. All animal work was approved by the University of Chicago Institutional Animal Care and Use Committee (IACUC).

Animal model and diet: At 4 weeks of age, FVB/N mice were separated and randomly assigned to either a control diet group (N=4) or a high-fructose diet group (N=4). The high-fructose diet matched the control group in all aspects except for the presence of 100 g/kg fructose which was replaced by corn starch (a polymer of glucose found in corn which is slowly digested to glucose) in the control diet. While HFCS is often produced from corn starch, the catabolism of fructose is much more rapid and known to have much more deleterious health outcomes than corn starch due to the separate catabolism of fructose to that of glucose, hand the low glycemic index of corn starch. These diets were purchased from Harlan Lab (Madison, WI). Figure 1 provides a list of nutrients and ingredients, respectively, for both control and high-fructose diets. Due to the differences in composition, the control diet (3.8 kcal/g) has slightly less caloric content by weight when compared to the high-fructose diet (3.9 kcal/g). Following studies, at 16 weeks of age mice were sacrificed by an overdose of isoflurane and cervical dislocation.

TD.140022 High Fructose Diet (60%kcals, Green)

Formula	g/Kg
Casein	200.0
L-Cystine	3.0
Sucrose	100.0
Fructose	529.386
Soybean Oil	70.0
Cellulose	50.0
Mineral Mix, AIN-93G-MX (94046)	35.0
Vitamin Mix, AIN-93-VX (94047)	10.0
Choline Bitartrate	2.5
TBHQ, antioxidant	0.014
Green Food Color	0.1

TD.94045 AIN-93G Purified Diet

Formula	g/Kg
Casein	200.0
L-Cystine	3.0
Corn Starch	397.486
Maltodextrin	132.0
Sucrose	100.0
Soybean Oil	70.0
Cellulose	50.0
Mineral Mix, AIN-93G-MX (94046)	35.0
Vitamin Mix, AIN-93-VX (94047)	10.0
Choline Bitartrate	2.5
TBHQ, antioxidant	0.014

Figure 1: Dietary information for control diet (TD.94045) and matched high-fructose diet (TD.140022). The only significant difference to the high-fructose diet compared to the control diet is the presence of fructose, which is replaced by corn starch in the control diet.

Metabolic Cage Testing: Metabolic cage testing was utilized at 16 weeks of age to further the metabolic understanding of the FVB/N mice under high-fructose conditions. Metabolic cages are used to measure circadian activity (energy expenditure) and feeding behavior of the mice through calculating food and water consumption, movement, and fuel utilization by measuring oxygen consumption and carbon dioxide production every thirty minutes. From these results, the average respiratory exchange ratio (RER) was calculated as a ratio of CO_2 produced versus O_2 consumed. Energy expenditure was further calculated as caloric value (CV) multiplied by O_2 consumed, where CV is equal to 3.815 + 1.232 x RER. After five days of acclimatization, measurements were taken every thirty minutes for two days. Heteroskedastic two-tailed t-tests were used to analyze the differences between control and fructose-fed FVB/N mice.

Dual-energy x-ray absorptiometry (DEXA): DEXA is an effective non-invasive approach to measure total body fat percentage as well as fat distribution among various depots. DEXA was also utilized to measure bone density of the mice. These experiments were completed in conjunction with the metabolic cage testing at 16 weeks of age. Heteroskedastic two-tailed t-tests were used to analyze the differences between control and fructose-fed FVB/N mice.

Glucose Tolerance Tests: To further the understanding of how a high-fructose diet effects metabolism in mice, glucose tolerance tests (GTTs) were conducted on the FVB/N mice after eight and twelve weeks on their respective diets. After an overnight fast, the GTTs were conducted via an intraperitoneal injection of twenty percent dextrose at a volume of 10 μL/g bodyweight. Blood glucose was measured pre-injection, post-injection, and at 15, 30, 60, and 120 minutes post injection. Blood, and subsequent serum via

ultracentrifugation, was also collected from the tail vain for insulin measurements via the ALPCO mouse ultrasensitive insulin ELISA (80-INSMSU-E01). Statistical differences in the GTTs were measured via area under the curve.

Histology: Following *in vivo* studies, epididymal white adipose tissue (WAT) from each mouse was placed in a histology cassette, formalin fixed, and paraffin embedded. During pathological sectioning, tissue was removed until a full face of tissue was found, and serial sections were taken to be placed on histological slides for subsequent analysis.

Perilipin Adipocyte Sizing: To directly analyze the effects of high-fructose feeding on adipose tissue in the mice, average adipocyte size was measured using an immunofluorescent perilipin antibody. Perilipin is a protein that associates with the surface of lipid droplets and can be used to estimate adipocyte size. For perilipin immunohistochemistry, 5 mm sections of WAT were cut and adhered to positively charged glass slides, de-waxed in xylene, and hydrated using graded ethanol washes. For heat-induced antigen retrieval, slides were placed within a steamer for 30 minutes in a citrate bath (EMS 10x Citrate Buffer, pH 6.0, Cat. # 64142-08). Immunostaining was performed using a 1:100 dilution of anti-perilipin antibody (Abcam, ab3526) with a donkey anti rabbit secondary antibody conjugated to DyLight® 594 (Abcam, ab96893). After images are taken using a FV1000 confocal microscope (Olympus), Cell Profiler imaging software was utilized to calculated average adipocyte size. Each biological replicate consisted of five technical replicates from sequential serial cut sections of adipose tissue. A heteroskedastic two-tailed t-test was used to analyze the differences in adipocyte size between control and fructose-fed FVB/N mice

C. Results

High-fructose feeding in FVB/N mice for twelve weeks is not associated with an increase in bodyweight, but there is an increase in bone-mineral density when compared to control-fed mice: At four weeks of age, mice were weaned and randomly assigned a high-fructose (N=4) or control diet (N=4). After twelve weeks of feeding, and at 16 weeks of age, the mice underwent DEXA analysis to investigate differences in diet on body weight and bone mineral density (BMD). This analysis assessed body weight, lean body mass, and body fat percentage (Figure 2). FVB/N mice on a high-fructose diet had a comparable weight to the mice fed the matched control diet after twelve weeks on diet (average body weight of 21.6 g +/- 0.59 for control fed mice compared to 21.6 g +/- 0.81 for the fructose-fed mice, p= 0.98). Similarly, there is no significant difference in lean body mass between the fructose-fed and control fed FVB/N mice after twelve weeks on diet (average lean body mass of 18.2 g +/- 0.48 for control fed mice compared to 18.6 g +/- 0.68 for their fructose-fed counterparts, p= 0.65). There is also no significant difference in body fat percentage between the two groups. (average body fat percentage of 15.6 % +/- 1.04 for the control fed FVB/N mice versus 13.7 % +/- 0.65 for the fructose-fed mice, p= 0.17). Despite no significant differences in body weight, lean body mass, or body fat percentage between the control and fructose-fed cohorts, there is a significant increase in femoral BMD in the fructose-fed mice after twelve weeks of high fructose feeding (Figure 3). There is an average BMD of 0.075 g/cm^2 +/- 0.001 for control animals, compared to 0.080 g/cm^2 +/- 0.0004 for the fructose-fed cohort (p= 0.02).

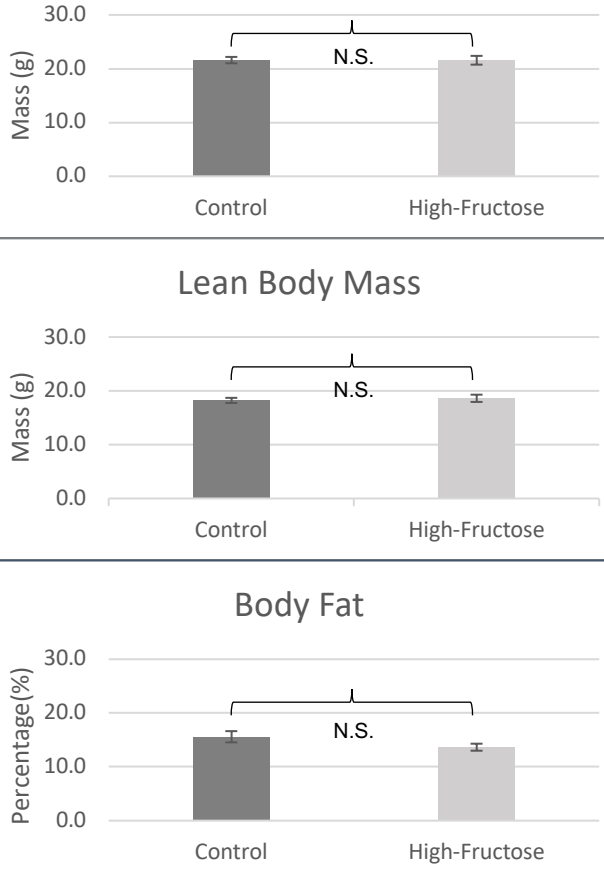


Figure 2: Body composition of control (N=4) and fructose-fed (N=4) mice after 12 weeks on diet. There is no significant difference between cohorts in weight (p=0.17), lean body mass (p=0.98) or percentage body fat (p=0.65).

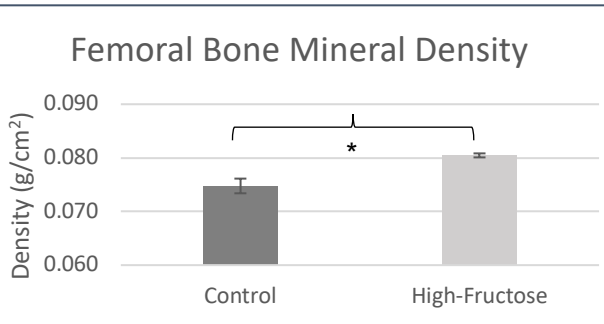


Figure 3: Right femur bone mineral density of control (N=4) and fructose-fed mice (N=4). There is a significant increase in bone mineral density in fructose-fed animals compared to controls (p=0.02).

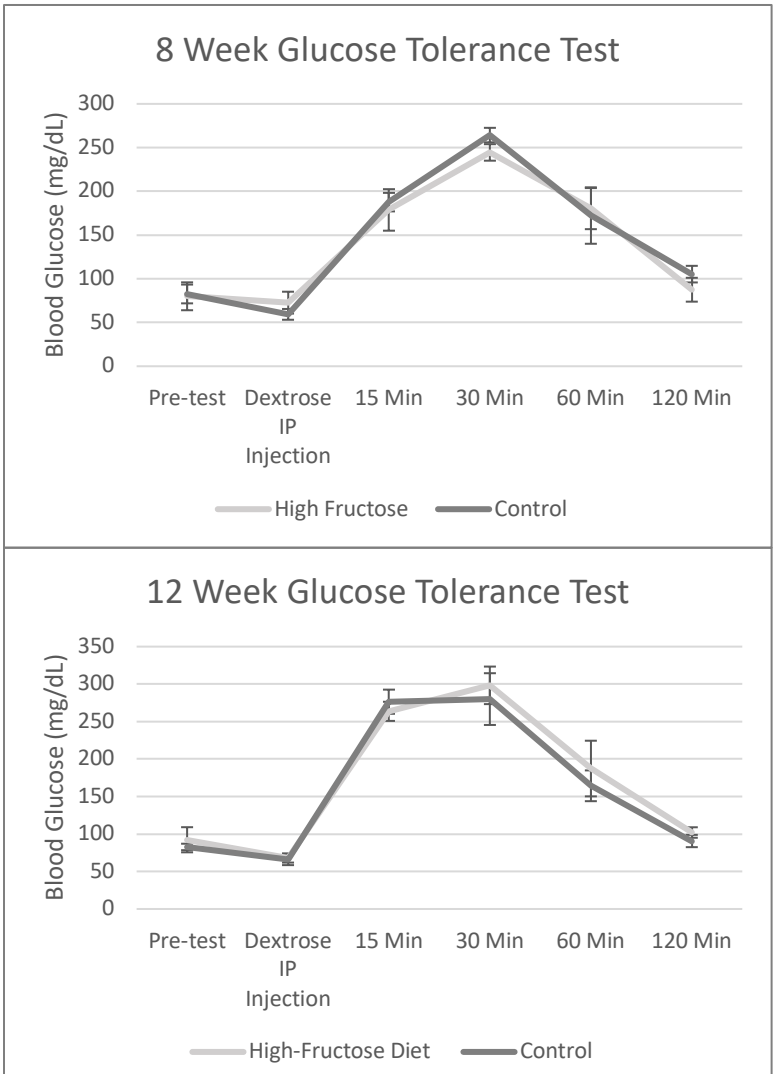


Figure 4: A glucose tolerance test was performed at both 8 and 12 weeks on diet for control (N=4) and fructose-fed (N=4) animals. There is no significant difference between groups as measured by area under the curve.

No differences in glucose tolerance after high-fructose feeding in FVB/N mice: After both eight and twelve weeks on diet GTTs were performed on FVB/N mice (Figure 4). After an overnight fast, an IP injection of dextrose was performed, and blood glucose was monitored. No significant difference in glucose tolerance was measured between control (N=4) and fructose (N=4) fed animals at either timepoint. There was also no change in serum insulin levels (data not shown).

After twelve weeks of high-fructose feeding FVB/N mice on a high-fructose diet have an increased adipocyte size when compared to their control-fed counterparts: Upon sacrifice, after twelve weeks of feeding and at sixteen weeks of age, epididymal WAT was fixed, paraffin embedded and placed onto histological slides. Each block of adipose tissue produced five serial cut technical replicates. These slides were used for the comparison of adipocyte size between control (N=4) and fructose-fed (N=4) cohorts. To do this a florescent perilipin antibody was utilized.

Perilipin is a protein which surrounds the lipid droplet, and lipid droplet size is a close estimation of adipocyte size. A confocal microscope captured these images and cell profile image analysis software was used to analyze the difference in adipocyte size between the control and fructose-fed cohorts.

Despite no measurable difference in body weight between these two groups, fructose-fed mice had a significant increase in adipocyte size when compared to the control-fed mice after twelve weeks of feeding (Figure 5). The control-fed mice had an average adipocyte size of 5260.5 μm^3 +/- 524.6, whereas fructose-fed mice had an average area of 8575.2 μm^3 +/- 682.5 ($p= 0.0005$).

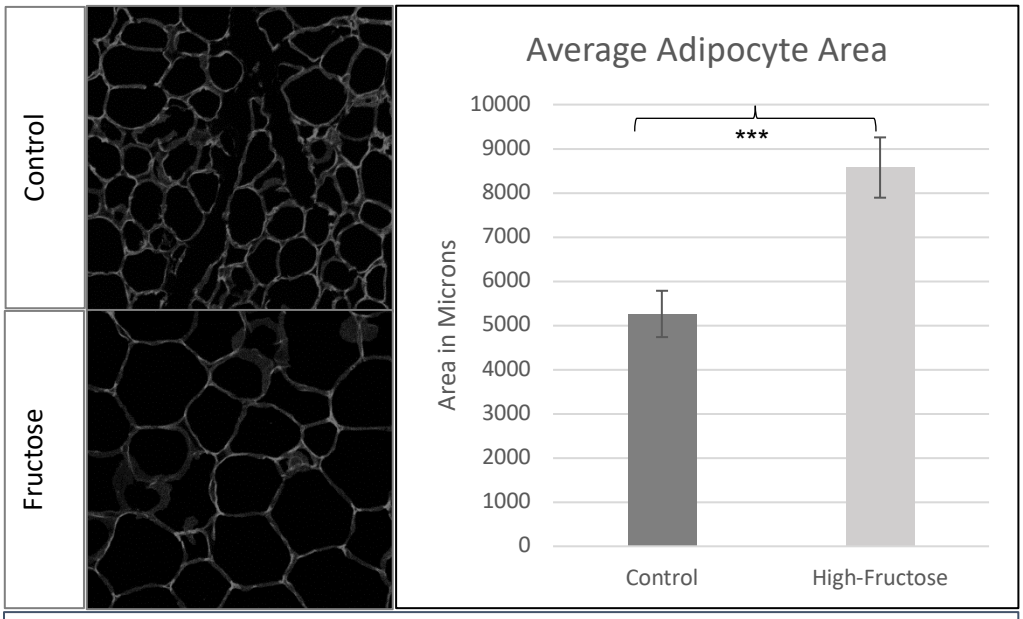


Figure 5: Average adipocyte area was measured for control (N=4) and fructose-fed animals (N=4). Each biological replicate consists of 5 technical replicates. Utilizing confocal microscopy (40x), perilipin IHC (ab3256, 1:100) was used to outline lipid droplets on slides of formalin fixed, paraffin embedded white adipose tissue from mice after 12 weeks on diet. Cell Profiler™ image analysis software was used to calculate average adipocyte size. Adipocytes from fructose-fed animals are significantly larger than adipocytes from control-fed mice (p=0.0005).

Metabolic cage data shows significant differences between control and fructose-fed FVB/N mice after twelve weeks of feeding: After twelve weeks of feeding, metabolic cages were utilized to discern more possible differences between the fructose (N=4) and control-fed (N=4) mice. Data from the metabolic cages allowed the analysis of food and water consumption, ambulatory movements, fuel utilization via RER, and energy expenditure. Food and water consumption were measured over a twelve-hour active period and found that fructose-fed animals consumed significantly more food and water than compared to their control-fed counterparts (Figure 6). Control-fed mice consumed an average of 1.1 g food +/- 0.07 whereas the fructose-fed mice consumed an average 1.6 g food +/- 0.03 over a twelve-hour active period for the animals (p= 0.0001). Furthermore, control-fed mice drink an average of 0.87 mL H20 +/- 0.06 over twelve hours, but the fructose-fed mice drink an average of 1.1 mL H20 +/- 0.07 (p= 0.02).

Fructose-fed animals also had a significant increase in ambulatory movements along both the X and Z axis, had a corresponding significant increase in energy expenditure, and also had a significant increase in energy expenditure (Figure 7). The number of ambulatory movements along the X and Z axis were measured over a period of twelve-hour period. Along the X axis, control diet fed mice conducted an average of 42089 counts +/- 1705, whereas the fructose-fed mice had an average of 107509 counts +/- 4331 ($p= 4.14e^{-8}$). Along the Z axis, control-fed mice conducted an average of 8309 counts +/- 443, but the fructose-fed mice had an average of 19163 counts +/- 682 ($p= 2.98e^{-9}$).

In correspondence with the increase in movement, the fructose-fed cohort also had an increase in energy expenditure. Control-fed mice had an average energy expenditure

of 0.37 kcal/hour +/- 0.003, where the fructose-fed mice had an average energy expenditure of 0.43 kcal/hour +/- 0.004 ($p= 2.55e^{-9}$).

Lastly, RER was also calculated as part of the metabolic cage testing after twelve weeks on diet. RER was calculated as the ratio of CO_2 production and O_2 consumption every thirty minutes. In general, RER values occur between 0.7 and 1.0 and correspond significantly with fuel utilization. A RER closer to 0.7 indicates that the predominant fuel source is lipid, whereas a RER closer to 1.0 is representative of carbohydrate being the predominant fuel source. Control-fed FVB/N mice had an average RER of 0.86 +/- 0.008, whereas the fructose-fed cohort had an average RER of 0.89 +/- 0.007 ($p= 0.008$).

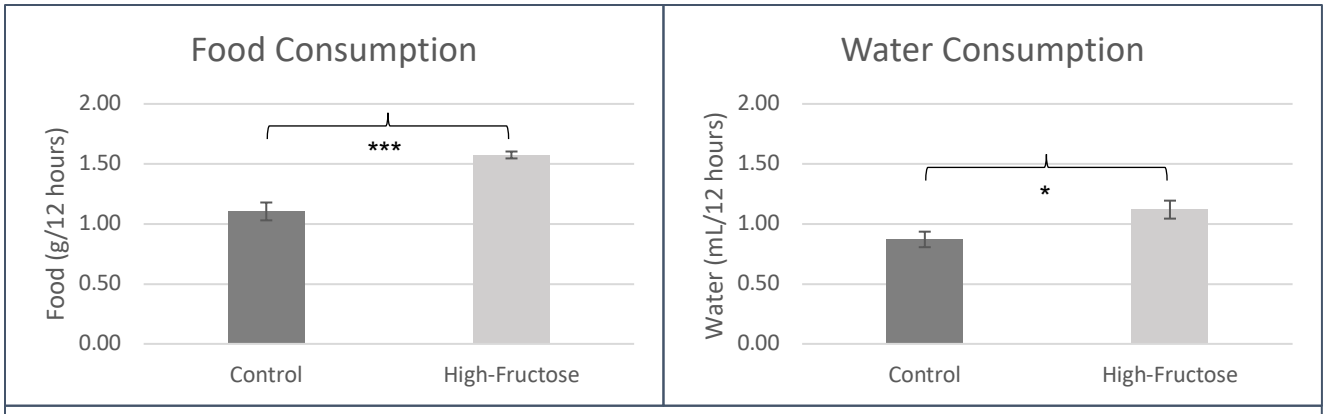


Figure 6: Total consumption of both food and water over a 12-hour period for control (N=4) and fructose-fed (N=4) mice. There is a significant increase in both food (p=0.0001) and water (p=0.02) consumption for fructose-fed animals when compared to controls.

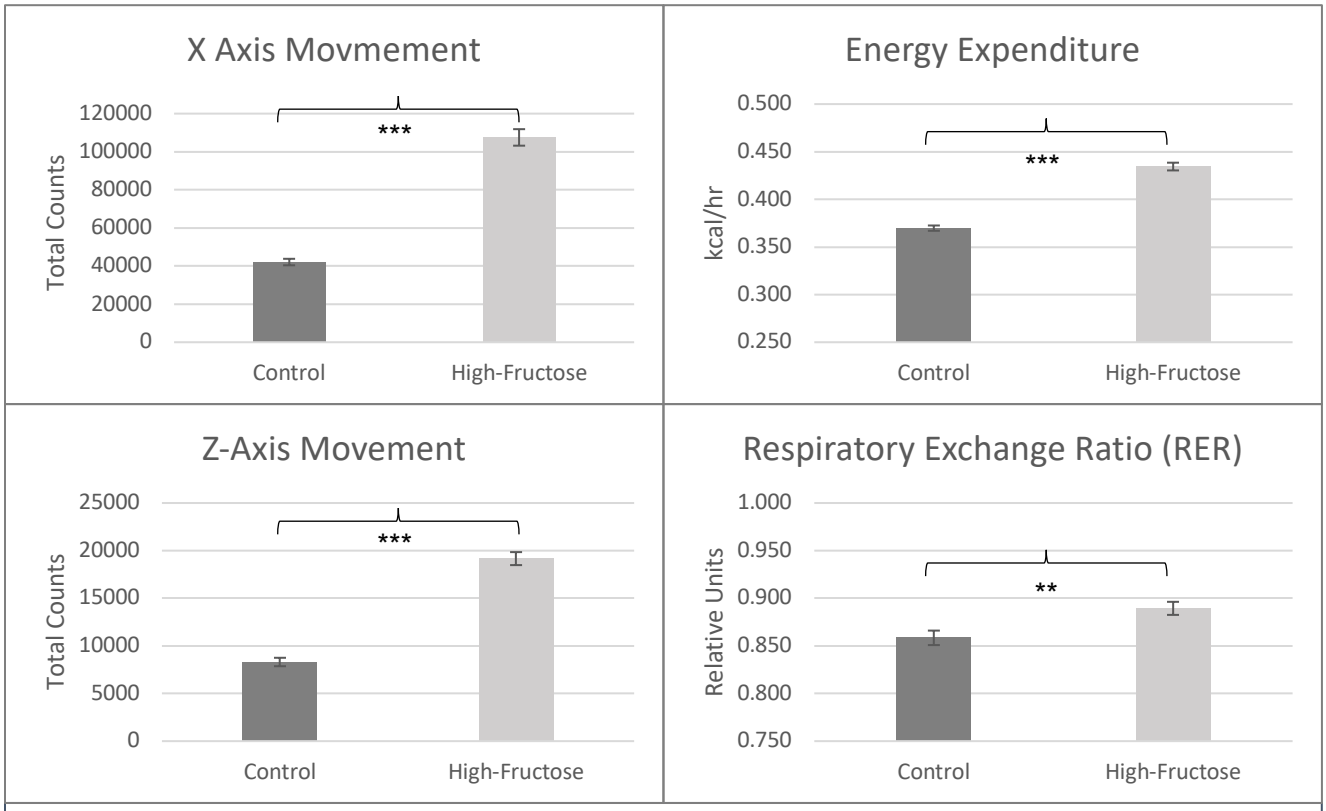


Figure 7: Total movement on the X and Z axes, energy expenditure, and respiratory exchange ratio was measured via metabolic cages while comparing control (N=4) and fructose-fed animals (N=4). The fructose cohort had a significant increase in movement along both the X ($p=4.1e^{-8}$) and Z axes ($p=3.0e^{-9}$) when compared to the control cohort. Fructose-fed animals also have a significant increase in energy expenditure ($p=2.6e^{-9}$) and RER ($p=0.008$) when compared to controls.

D. Discussion

The present study examined the metabolic outcomes of high-fructose feeding in FVB/N mice after twelve weeks on diet, which corresponds to sixteen weeks of age. This is only one of a few studies to assess the effects of long-term high-fructose feeding, as well as on FVB/N mice. These experiments are not only essential to assess the impact of long-term high-fructose feeding, but also to investigate how a fructose-enriched diet specifically effects FVB/N mice. As it is imperative to have a firm understanding of metabolic outcomes of a specific mouse background before further experimentation, these studies will provide the necessary information needed for further experiments in chapter III.

Some have argued that FVB/N mice are resistant to diet-induced obesity – especially when compared to often used C57BL/6 mice or other obesity prone mouse backgrounds [38]. Congruent with this, this study found that the FVB/N mice fed a high-fructose diet for twelve weeks had no significant change in body weight when compared to the control diet fed cohort (Figure 2). Furthermore, there was also no significant change in glucose tolerance between the fructose and control-fed cohorts after either eight or twelve weeks of feeding (Figure 4). These findings uniquely allow for the investigation of high-fructose feeding in FVB/N mice without the confounding effects of obesity and systemic insulin resistance.

Despite no change in weight or glucose tolerance between control and fructose-fed FVB/N mice, significant metabolic differences were seen in peripheral tissues

between these two cohorts. There is a significant increase in both epididymal adipocyte size and in BMD in the fructose-fed mice (Figures 6 and 4).

The increase in adipocyte size is not surprising, even without the presence of obesity, as a high-fructose diet is well established to enhance lipogenic pathways [39]. Starting in the liver, the process of fructolysis is completely unregulated, bypassing the rate-limiting steps of glycolysis. This uncontrolled metabolism of fructose leads to the buildup of many metabolic intermediates, and without increases exercise most of the excess carbon is shunted towards lipid production, as only so much energy can be stored as glycogen. While fatty liver is common with prolonged fructose intake, only so much ectopic lipid can be stored before large amounts VLDL is released to be stored in peripheral adipose depots [40]. Therefore, even in the absence of obesity, after twelve weeks of high-fructose feeding it is not surprising to see increases to adipocyte size as lipid continues to be produced and stored at heightened rates.

As mentioned earlier, these fructose-fed FVB/N mice also exhibit an increase in BMD when compared to the control-fed mice after twelve weeks of feeding. As this is previously been reported in long-term high-fructose rat studies, this outcome was hypothesized; however, the mechanism by which a high-fructose diet increases BMD is yet to be fully elucidated [41]. Furthermore, the previous rat studies postulated the increase in BMD was due to an increase in high density lipoprotein (HDL) or simply caused by an increase in body mass [42]. In the case of this study, the FVB/N mice on a high-fructose diet had no change in body weight, and the levels of HDL were not measured. Other groups have suggested that the aforementioned changes to adipose tissue and lipid metabolism could be a potential mechanism by which BMD is increased.

It has been well established that adipose tissue is an endocrine organ, and that adipokines and fatty acids in circulation that were secreted from adipocytes can impact osteoblast differentiation [43]. While these pathways are not something this project was able to investigate, there are other, more direct, factors which may also be influencing BMD. Prolonged mechanical stress, such as increased movement, is also known to increased BMD [44]. As these FVB/N mice fed a high-fructose diet for twelve weeks had a significant increase in both movement and energy expenditure, with no change in body weight, we propose that the increase in BMD is caused by an increase in movement, and subsequent mechanical stress. Further studies are required to fully understand how a sustained high-fructose diet causes significant increases in BMD.

The fructose-fed FVB/N mice were indeed shown to have a significant increase in energy expenditure and movement compared to their control-fed counterparts (Figure 7). With the excess in dietary fructose these mice have an increase in movement, energy expenditure, and RER. While the increase in movement may provide the increased mechanical stress to increase BMD, it also provides some rational to why these mice have not gained weight after twelve weeks of high-fructose feeding, despite having a significant increase in food consumption compared to the control-fed mice (Figure 6). Moreover, the high-fructose diet (3.9 kcal/g) had slightly more calories when compared to the control diet (3.8 kcal/g) due to the incorporation of fructose into the food. This corresponds to the average fructose-fed mouse consuming an extra 2.06 kcals every twelve hours when compared to the control-fed cohort. The significant increase in energy expenditure in the fructose-fed mice only accounts for an increase of 0.72 kcals every twelve hours. As more ATP is utilized in the catabolism of fructose compared to that of

glucose, the simple metabolism of a high-fructose diet may be responsible for the increase in caloric intake with no change in average body-weight between the fructose and control-fed cohorts [45]. As the metabolism of fructose can diverge and many directions, and some of its byproducts, such as lactate, may be utilized for ATP generation, further studies into the bioenergetics of fructose metabolism are required to conclude that the metabolism of fructose is responsible for the increased caloric intake.

Interestingly, in opposition to these data, many previous studies have concluded that a high-fructose diet actually decreases energy expenditure, or any increase in energy expenditure is due to increased thermogenesis [46, 47]. From this study it is evident that FVB/N mice fed a high-fructose diet for twelve weeks have a significant increase in RER and movement, which suggests an increase in energy expenditure. However, for this study, energy expenditure was calculated as a function of RER and oxygen consumption, and not directly, so it is possible that the increase in energy expenditure is mostly a function of RER. In general, RER does increase as a function of energy expenditure, as increased exertion will make carbohydrate the preferred fuel source of the body over lipid which predominates at rest. In this study, however, under such high-fructose conditions, carbohydrate may be the predominant fuel source regardless of energy expenditure [48].

Overall, this study has demonstrated that twelve-week high-fructose feeding in FVB/N mice does not cause a significant difference in body weight or glucose tolerance when compared to controls; however, metabolic differences can be seen, as is evident by the increased adipocyte size. For these reasons, the FVB/N mouse will be ideal to interrogating metabolic pathways disrupted by high-fructose feeding, without the confounding influences of obesity, which can contribute to metabolic differences by itself.

Throughout chapter III, the impact of high-fructose feeding on hepatic metabolic pathways will be more thoroughly investigated in the FVB/N mouse model.

Chapter III

Hepatic fructose metabolism in FVB/N mice

A. Introduction

The negative health outcomes of high-fructose feeding have begun to be understood, yet the underlying mechanisms have yet to be full elucidated. From human studies, it is evident as obesity rates continue to climb, so do the amounts of fructose consumed – mostly through the incorporation of HFCS into sweetened and processed foods [3]. High-fructose diets have been implicated in the development of numerous aspects of metabolic disease, such as diabetes, fatty liver, and high blood pressure [23, 30]. To better understand these deleterious effects of a high-fructose diet, many animal studies have been implemented to interrogate the mechanisms and associated health outcomes of high-fructose feeding [27, 28, 31, 34, 38, 49]. While informative, many of these studies were short in duration (<4 weeks), making it hard to conclude how prolonged fructose consumption alters metabolism over time and how these changes may be associated with many of the negative health outcomes.

Throughout chapter II the metabolic health outcomes of long-term high-fructose feeding in FVB/N mice were interrogated. It was concluded that these mice are resistant to high-fructose-diet-induced obesity, allowing for long-term study of high-fructose feeding without the confounding effects of obesity which create distinct metabolic profiles of its own. Throughout chapter III, the most will be made of this phenotype as the differential effects of short and long-term high-fructose feeding are interrogated in FVB/N

mice for the first time, and longer-term high-fructose feeding studies in any strain of mice are also sparsely described in the published literature.

Fructose consumed in low concentrations, such as fructose found naturally in fruits, is easily digested in the small intestines into glucose, which is easily catabolized through the normal glycolytic pathway once it reaches the liver through the hepatic portal vein [15, 17]. The gut metabolism of fructose is thought to protect the liver from many of the harmful effects of fructolysis, but in higher concentrations the small intestine cannot convert all of the fructose to glucose and much of the fructose is left to be metabolized by the liver.

In the liver fructose is rapidly phosphorylated by KHK followed by an AldoB catalyzed reaction which further metabolizes the fructose molecule into dihydroxyacetone-phosphate (DHAP) and glyceraldehyde. From here triokinase / FMN cyclase (TKFC) and pyruvate kinase (PKL) catalyze the formation of glyceraldehyde-3-phosphate and pyruvate, respectively. Many of these intermediates can be shunted towards gluconeogenesis to replenish glycogen stores; however, in the absence of exercise or depleted glycogen stores, many of these remaining carbon intermediates are utilized for de novo lipogenesis and the production of fatty acids [14, 50]. Furthermore, this process of hepatic fructolysis is completely unregulated, so it will attempt to continue as long as fructose continues to be ingested. In sedentary individuals this process can lead to excessive lipid accumulation, both in the liver and in peripheral tissues [29].

In addition to the intensified production of fatty acids, which may be the root cause of many negative health outcomes associated with high-fructose feeding, the uncontrolled hepatic fructolysis can also deplete ATP pools and other organic cofactors which drive

many of these metabolic processes [21]. As previously states, KHK readily phosphorylates fructose, yet AldoB is the rate-limiting step of fructolysis, leading to a build-up of hepatic fructose-1-phosphate after the consumption of fructose. As KHK requires ATP to function, pools of organic cofactors can be significantly depleted after high-fructose feeding. The impact of this with prolonged high-fructose feeding is yet to be properly interrogated, yet the potential to impact hepatic metabolism is evident if pools of organic cofactors continue to be depleted.

In addition to the direct catabolic pathway of fructose, the consumption of fructose is known to alter metabolism is several ways. Of interest to this study, dietary fructose has been shown to significantly alter both hepatic glucocorticoid signaling and the production of fibroblast growth factors. Furthermore, both of these pathways have been shown to be influential in regulating the development of metabolic syndrome and other related diseases; however, more work is required to fully understand how long-term high-fructose feeding can alter these pathways overtime to lead to the progression of metabolic disease [49, 51-55].

Glucocorticoids are stress hormones that respond to both perceived and dietary stressors, which circulate throughout the body to influence numerous metabolic processes. In mice, the predominant active glucocorticoid is corticosterone which is released via the hypothalamic-pituitary-adrenal (HPA) axis in response to numerous stressors. Glucocorticoids are generally released into circulation in their inactive form – 11-dehydrocorticosterone (11-DHC) for rodents – and are only activated through an enzymatic process in the presence of 11β-hydroxysteroid dehydrogenase 1 (11β-HSD1) [56].

Excess of glucocorticoid signaling is associated with severe metabolic dysfunction and the onset of obesity [57]. Interestingly, dietary fructose is proposed to elevate glucocorticoid signaling through increased 11β-HSD1 activity, which generates the physiologically active glucocorticoid, corticosterone [53]. To our knowledge, the ability of fructose to stimulate 11β-HSD1 is yet to be assessed in FVB/N mice.

Intriguingly, recent data indicates that glucocorticoid activity can stimulate the release of Fibroblast Growth Factor – 21 (FGF-21) – an important hepatokine involved in energy homeostasis – and that acute high-fructose feeding is also associated with an increase in FGF-21 [58]. Although much recent work has shown that a fructose challenge can stimulate FGF-21 acutely, more research is required to investigate the impact of long-term fructose feeding on the production of FGF-21.

Over the past fifteen years FGF-21 has been described as an important metabolic regulator and was originally characterized for its role in the fasting response, where during a prolonged fast PPARα drives the expression of FGF-21 [59, 60]. Subsequently, FGF-21 has been found to act throughout the body to increase energy expenditure, heighten insulin sensitivity and glucose uptake, induce thermogenesis, and even alter feeding behavior and macronutrient preference to cause aversion to sweet foods [61]. Due to the positive effects of FGF-21 throughout the body, there have been attempts to use it as a drug to fight metabolic disease. Paradoxically, obese populations are already found to have heightened levels of circulating levels of FGF-21 and are thought to become resistant to the naturally circulating hormone [62, 63]. Of note, acute ingestion of dietary fructose is shown to elicit a strong increase in circulating FGF-21, which is thought to try to protect the body from the negative metabolic responses to high-fructose feeding [64].

ChREBP activation, in conjunction with PPARα signaling, is thought to stimulate this strong response of FGF-21 to acute fructose consumption [65]. Although the short-term response of FGF-21 to high-fructose feeding is well described, the long-term adaptive responses to high-fructose feeding and their impact on FGF-21 had previously not been intensively interrogated.

The objective of this study is to interrogate the role of a high-fructose diet on hepatic metabolism. Many recent studies have investigated the role of an acute fructose challenge to alter metabolism; however, to better understand how prolonged fructose-feeding can lead to the progression of multiple metabolic diseases, more research is required. In this study, we sought to answer some of these questions by investigating hepatic fructose metabolism at two separate time points: a 48-hour study and a twelve-week study. After 48 hours of high-fructose feeding in FVB/N mice, this analysis found similar results to the majority of published literature, where high-fructose feeding is associated with an increase in both fructolytic and lipogenic hepatic pathways, but also a significant increase in the production of FGF-21. Interestingly, after twelve weeks of high-fructose feeding, these same pathways are no longer significantly upregulated.

B. Materials and methods

Animal care: This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. All animal work was approved by the University of Chicago Institutional Animal Care and Use Committee (IACUC).

Animal model and diet: FVB/N mice were used throughout all studies. For this study, mice were testing in two arms: a twelve-week study similar to chapter II, and a 48-hour study. For the twelve-week study, FVB/N mice were separated at four weeks of age and randomly assigned to either a control diet group (N=15) or a high-fructose diet group (N=11). For the 48-hour study, FVB/N mice were not assigned to the high-fructose (N=4) or control (N=4) cohorts until two days before 16 weeks of age.

The high-fructose diet matched the control group in all aspects except for the presence of 100 g/Kg fructose which was replaced by corn starch in the control diet. Due to the differences in composition, the control diet (3.8 kcal/g) has slightly less caloric value by weight when compared to the high-fructose diet (3.9 kcal/g). These diets were purchased from Harlan Lab (Madison, WI). Figure 1 provides a list of nutrients and ingredients, respectively, for both control and high-fructose diets. At 16 weeks of age mice were sacrificed by an overdose of isoflurane and cervical dislocation.

Hepatic gene expression: For the analysis of hepatic gene expression, the liver was immediately frozen on dry ice after sacrifice and stored at -80^{o}C until RNA was extracted. The liver was homogenized utilizing the Next Advance Bullet Blender tissue homogenizer and the correspond Green Rino RNA lysis kit. Following tissue homogenization, RNA was extracted using the Omega E.Z.N.A Total RNA Kit II (R6934-01). Following RNA extraction, iScript Reverse Transcription Supermix for RT-qPCR (BIO RAD, 1708840) was used to produce cDNA. Gene expression for genes of interest was measured with quantitative real time polymerase chain reaction (qRT-PCR). 18S Ribosomal RNA was used as a house-keeping gene throughout all measured genes. Statistical analysis

comparing control-fed and fructose-fed cohorts was conducted on log-transformed dCT values with heteroskedastic two-tailed t-tests.

Blood serum collection and metabolite measurement: Upon animal sacrifice, blood was collected via cardiac puncture. After allowing blood to clot for 30 minutes, serum was separated with centrifugation for 10 minutes at 2,000 x g. Samples were stored at -80^{o}C until experimentation. Serum concentration of FGF-21 was measured with Abcam's fluorescent mouse FGF-21 ELISA kit (ab229382) from the stored samples. Heteroskedastic two-tailed t-tests were used to examine the statistical difference in serum FGF-21 concentration between control and fructose-fed cohorts.

C. Results

Increase in fructolysis genes after a 48-hour high-fructose diet, but not after twelve weeks on diet: Expression of key fructolysis genes were measured upon sacrifice, after a 48-hour diet and a twelve-week diet - both at sixteen weeks of age. Gene expression of KHK, AldoB, TKFC, LDHA, and PKL were all measured at both 48-hours and 12-weeks on diet, for both the control and fructose fed cohorts (Figure 8).

After 48-hours of feeding there was a significant increase for all measured genes in the fructose-fed mice compared to the control cohort. There was a 2.51-fold increase of KHK in fructose-fed mice compared to their control-fed counterparts after 48 hours of feeding ($p= 0.02$). For AldoB there was a 3.58-fold increase in the fructose-fed FVB/N mice after 48 hours compared to the control-fed animals ($p= 0.0009$). In TKFC expression there was a 2.14-fold increase for the fructose-fed cohort when compared to the control

mice (p= 0.04). LDHA was also increased in fructose-fed animals, with a 2.29-fold increase in expression compared to the control-fed mice (p= 0.007). Lastly, PKL was also increased 2.92-fold in the fructose-fed cohort compared to the control animals (p= 0.005). Although almost all studies on high fructose feeding have occurred on the acute time scale, similar to the 48-hour arm of the study, few studies have been conducted more chronically, and none to our knowledge in the obesity resistant FVB/N strain of mice.

In our studies, opposed to the 48-hour feeding results, after twelve weeks of feeding there is no significant difference between the control and fructose-fed cohorts for any of the measured fructolytic genes. After twelve weeks of high-fructose feeding there is a 1.36-fold increase in KHK, 1.53-fold increase in AldoB, 1.15-fold increase in TKFC, 1.14-fold increase in LDHA, and 1.27-fold increase in PKL for fructose-fed cohorts when compared to their control-fed counterparts (p= >0.05 for all relationships).

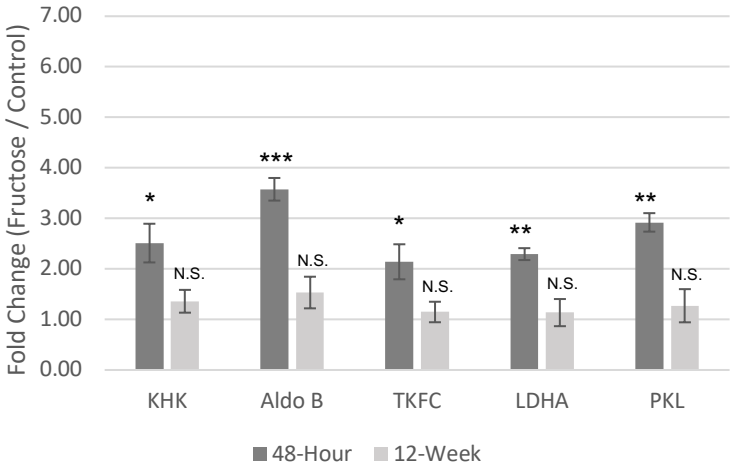


Figure 8: Major fructolysis genes were analyzed via qRT-PCR after 48 hour (Control N=4, High-Fructose N=4) and 12 weeks (Control N=15, High-Fructose N=11) on diet in both control and fructose-fed mouse liver. Fold-change in gene expression of fructose-fed mice compared to control fed mice was calculated. After 48-hours there is a significant increased in all measured fructolysis-related genes in fructose-fed animals compared to controls (KHK p=0.02, Aldo B p=0.0009, TKFC p=0.04, LDHA p=0.007, PKL p=0.005). There is no significant difference in any measured genes between control and fructose-fed animals after 12 weeks on diet.

Increase in adipogenic genes after a 48-hour high-fructose diet, but not after twelve weeks on diet: Expression of adipogenesis-related genes were measured upon sacrifice, after a 48-hour diet and a twelve-week diet - both at sixteen weeks of age. Gene expression of Peroxisome Proliferator-Activated Receptor Gamma (PPARγ), Acetyl-CoA Carboxylase (ACC), ATP Citrate Lyase (ACLY), Acyl-CoA Synthetase Short Chain Family Member 2 (ACSS2), and Fatty Acid Synthase (FASN) were all measured at both 48-hours and 12-weeks on diet, for both the control and fructose fed cohorts (Figure 9).

After 48-hours of feeding there was a significant increase for all measured genes in the fructose-fed FVB/N mice compared to the control cohort. After 48 hours of high-fructose feeding PPARγ is increased 2.18-fold compared to control-fed mice ($p= 0.02$). Expression of ACC is increased 3.51-fold in the fructose-fed cohort compared to controls ($p= 0.03$). Under high-fructose conditions ACLY is increased 4.74-fold when compared to their control-fed counterparts ($p= 0.006$). Expression of ACSS2 is increased 4.87-fold in fructose-fed animals in comparison to the control-fed mice ($p= 0.002$). Lastly FASN is also increased 2.99-fold in fructose-fed FVB/N when compared to the control-fed mice ($p= 0.03$).

Opposed to the 48-hour feeding results, after twelve weeks of feeding there is no significant difference between the control and fructose-fed cohorts for any of the measured adipogenic genes. For the fructose-fed FVB/N mice fed a high-fructose diet there is a 1.37-fold increase in PPARγ, a 1.59-fold increase in ACC, a 1.16-fold increase in ACLY, a 1.21-fold increase in ACSS2, and a 1.52-fold increase in FASN when compared to the control-fed mice ($p= >0.05$ for all relationships).

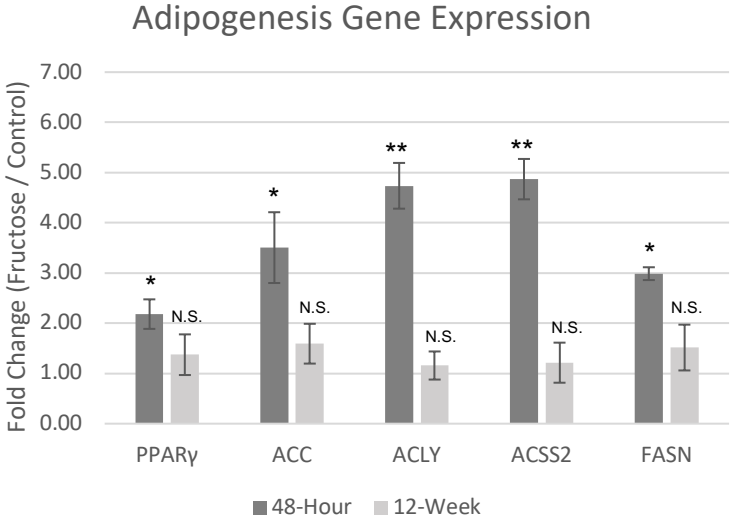


Figure 9: Adipogenesis-related genes were analyzed via qRT-PCR after 48 hour (Control N=4, High-Fructose N=4) and 12 weeks (Control N=15, High-Fructose N=11) on diet in both control and fructose-fed mouse liver. Fold-change in gene expression of fructose-fed mice compared to control fed mice was calculated. After 48-hours there is a significant increased in all measured adipogenesis-related genes in fructose-fed animals compared to controls (PPARγ p=0.02, ACC p=0.03, ACLY p=0.005, ACSS2 p=0.002, FASN p=0.03). There is no significant difference in any measured genes between control and fructose-fed animals after 12 weeks on diet.

Increase in FGF-21 related genes after a 48-hour high-fructose diet; however, expression between control and fructose-fed cohorts is unchanged after twelve weeks on diet: Expression of FGF-21-related genes were measured upon sacrifice, after a 48-hour diet and a twelve-week diet - both at sixteen weeks of age. Gene expression of FGF-21, β-Klotho, and FGFr1 were all measured at both 48-hours and 12-weeks on diet, for both the control and fructose fed cohorts (Figure 10).

After 48-hours of feeding there was a significant increase for all measured genes in the fructose-fed mice compared to the control cohort. The FVB/N mice fed a high-fructose diet expressed a 5.92-fold increase in FGF-21 ($p= 0.006$), a 1.70-fold increase in β-Klotho ($p= 0.02$), and a 1.40 fold in FGFr1 ($p= 0006$) when compared to the control-fed cohorts after 48 hours.

Opposed to the 48-hour feeding results, after twelve weeks of feeding there is no significant difference between the control and fructose-fed cohorts for any of the measured FGF-21 related genes. The high-fructose diet-fed mice had a 0.65-fold change in FGF-21, a 1.09-fold change in β-Klotho, and a 0.78-fold change in FGFr1 when compared to the control-fed animals after twelve weeks of feeding ($p= >0.05$ for all relationships).

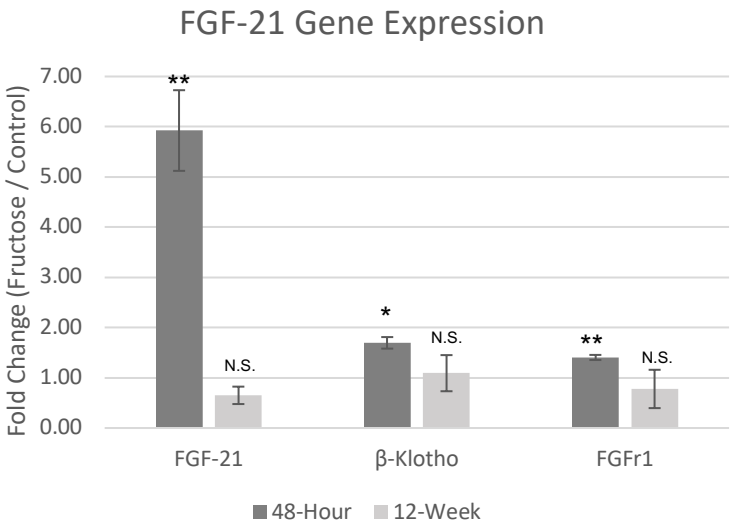
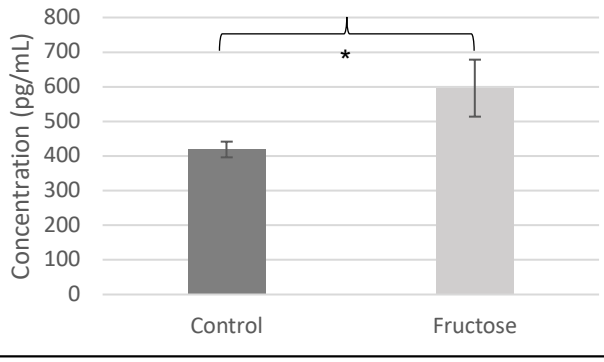


Figure 10: FGF-21-related genes were analyzed via qRT-PCR after 48 hour (Control N=4, High-Fructose N=4) and 12 weeks (Control N=15, High-Fructose N=11) on diet in both control and fructose-fed mouse liver. Fold-change in gene expression of fructose-fed mice compared to control fed mice was calculated. After 48-hours there is a significant increased in all measured FGF-21-related genes in fructose-fed animals compared to controls (FGF-21 p=0.006, β-Klotho p=0.02, FGFr1 p=0.007). There is no significant difference in any measured genes between control and fructose-fed animals after 12 weeks on diet.

Serum levels of FGF-21 correspond with gene expression results at both 48-hour and twelve weeks on diet: Upon sacrifice blood serum was collected from each mouse via cardiac puncture and subsequent centrifugation. To assess the circulating levels of FGF-21 an ELISA was conducted on each serum sample. The relative difference in serum levels of FGF-21 between fructose and control-fed FVB/N mice corresponded with the hepatic gene expression of FGF-21 at both 48-hours and twelve weeks on diet (Figures 11 and 12). As hepatic production of FGF-21 is the main source of circulating hepatokine it is not surprising that the hepatic expression correlates with circulating levels; however, to our knowledge this is the first study to interrogate the relationship between fructose consumption and FGF-21 production at multiple time-points.

After 48-hours of high-fructose feeding in FVB/N mice there is a significant increase in the concentration of circulating FGF-21 when compared to the control-fed cohort, and this difference correlates with the hepatic gene expression in the same study. After 48-hours on diet the control-fed FVB/N mice had an average FGF-21 blood serum concentration of 418.8 pg/mL +/- 22.7, whereas the fructose-fed mice had an average concentration of 596.0 pg/mL +/- 82.3 ($p= 0.04$).

In contrast, after twelve weeks of high-fructose feeding, there is a significant decrease in the concentration of circulating FGF-21 when compared to the control fed FVB/N mice. Again, these relative concentrations in circulations correspond with the relative hepatic expression of FGF-21 after twelve weeks on diet. These control-fed FVB/N mice had an average FGF-21 blood serum concentration of 738.2 pg/mL +/- 127.3, whereas the fructose-fed mice had an average concentration of 434.4 pg/mL +/- 51.2 ($p= 0.03$).



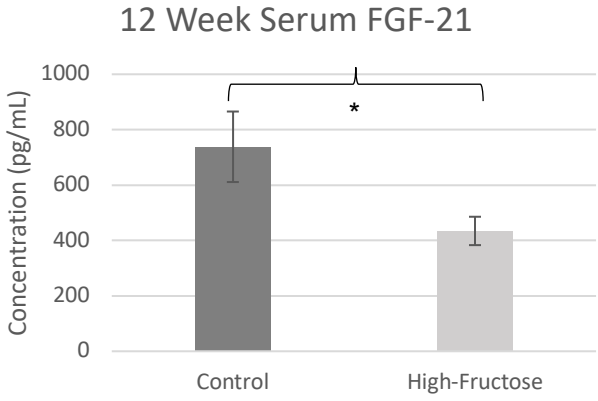


Figure 11: Serum FGF-21 was measured via ELISA (ab229382). After 48 hours on diet fructose-fed (N=8) mice had a significantly higher serum concentration of FGF-21 when compared to control-fed (N=8) animals (p=0.04). After 12 weeks on diet fructose-fed (N=26) animals had significantly lower serum concentration of FGF-21 when compared to control-fed (N=26) animals (p=0.03).

Increase in glucocorticoid signaling genes after a 48-hour high-fructose diet, but not after twelve weeks on diet: Expression of glucocorticoid-related genes were measured upon sacrifice, after a 48-hour diet and a twelve-week diet - both at sixteen weeks of age. Gene expression of 11β-HSD1 and SGK1 were measured at both 48-hours and 12-weeks on diet, for both the control and fructose fed cohorts (Figure 12).

After 48-hours of feeding there was a significant increase in 11β-HSD1 and SGK1 gene expression for the fructose-fed mice compared to the control cohort. The fructose-fed animals had 1.56-fold increase in 11β-HSD1 ($p= 0.03$) and a 1.84-fold increase in SGK1 ($p= 0.004$) when compared to the control-fed cohort after 48-hours on their respective diets.

Opposed to the 48-hour feeding results, after twelve weeks of feeding there is no significant difference between the control and fructose-fed cohorts for either 11β-HSD1 or SGK1. The fructose-fed FVB/N mice had a 1.10-fold change in 11β-HSD1 and a 1.14-fold change in SGK1 ($p= >0.05$ for all relationships).

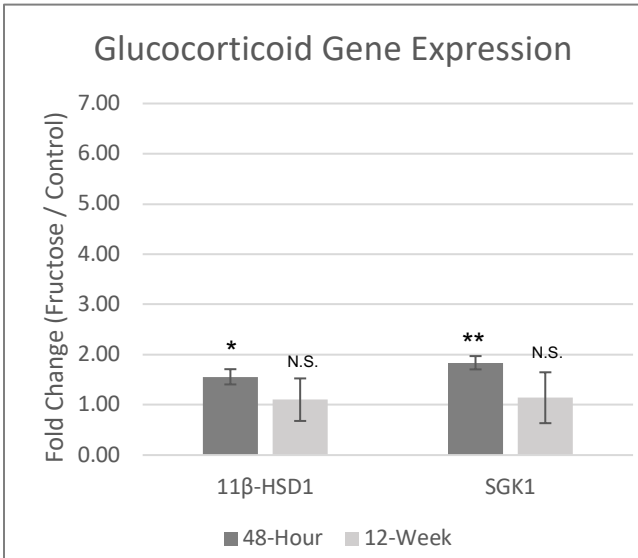


Figure 12: Glucocorticoid-related genes were analyzed via qRT-PCR after 48 hour (Control N=4, High-Fructose N=4) and 12 weeks (Control N=15, High-Fructose N=11) on diet in both control and fructose-fed mouse liver. Fold-change in gene expression of fructose-fed mice compared to control fed mice was calculated. After 48-hours there is a significant increased in all measured glucocorticoid-related genes in fructose-fed animals compared to controls (11β-HSD1 p=0.03, SGK1 p=0.004). There is no significant difference in any measured genes between control and fructose-fed animals after 12 weeks on diet.

Significant changes to adipose tissue lipogenic gene expression after 12-weeks high-fructose feeding: Upon sacrifice, epididymal WAT was flash frozen and stored at -80 C until RNA could be extracted for gene expression analysis via qRT-PCR. Major adipogenic genes including PPARγ, ACC, ACLY, ACSS2, and FASN were analyzed and shown as fold-change in gene expression between the control (N=10) and fructose-fed (N=10) FVB/N mice.

There was a significant increase in the WAT expression of PPARγ with a 4.9-fold increase in expression within the fructose-fed mice when compared to the corresponding control-fed cohorts ($p = 0.03$). In contrast there was a significant decreases in the WAT expression of all measured adipogenic enzymes, including a 0.34-fold change in ACC ($p = 0.03$), a 0.17-fold change in ACLY ($p = 0.04$), a 0.24-fold change in ACSS2 ($p = 0.02$), and a 0.34-fold change in FASN ($p = 0.02$).

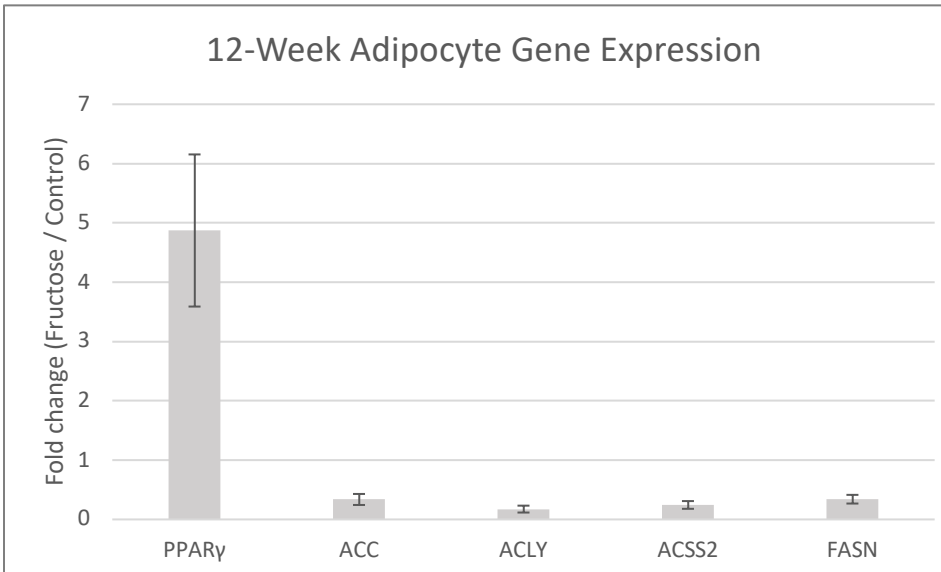


Figure 13: Adipogenesis-related genes were analyzed via qRT-PCR after 12 weeks (Control N=10, High-Fructose N=10) on diet in both control and fructose-fed mouse liver. Fold-change in gene expression of fructose-fed mice compared to control fed mice was calculated. After 12-weeks there is a significant increase in PPARγ gene expression, but all other measured adipogenesis-related genes are significantly decreased in fructose-fed animals compared to controls (PPARγ p=0.03, ACC p=0.03, ACLY p=0.04, ACSS2 p=0.02, FASN p=0.02).

D. Discussion

Over the past few years, a significant amount of research has been conducted to better understand the metabolism of fructose and its relation to metabolic disease. Many of these studies were short-term studies or were at least only conducted at a single time-point where changes in fructose metabolism and related pathways could accessed as they change with the continued stress of high-fructose feeding. Knowing how these metabolic pathways can be altered with continued insult is imperative to understanding how a steady diet may lead to progression of multiple diseases.

To better understand these fructolytic pathways and how they may translate to disease progression, throughout chapter III the differences between short (48-hour) and long-term (12-week) high-fructose feeding were interrogated in FVB/N mice. To our knowledge this is one of the first studies to investigate changes to they key metabolic pathways under high-fructose conditions at multiple time-points, and especially in a obesity-resistant FVB/N mouse model which allows for a dietary investigation without the confounding effects associated with obesity or insulin resistance.

Initially, the hepatic expression of key fructolytic genes were investigated, with KHK catalyzing the initial phosphorylation of fructose, and AldoB catalyzing the rate-limiting step of fructolysis. TKFC, LDHA, and PKL are also associated with the catabolism of fructose lower in the metabolic pathways. Many previous studies, which have generally been short in duration, have shown all of these genes to be significantly upregulated [27, 39]. This is not unexpected as the expression of enzymes that are increasingly utilized are upregulated in many cases – at least in the short term.

Indeed, in these studies, FVB/N mice fed a high-fructose diet for 48-hours also show a significant upregulation in all these critical fructolytic genes when compared to the control-fed cohort. As an increased fructose consumption begins to be catabolized there is a corresponding increase in the expression of critical enzymes. Interestingly, however, these studies have, for the first time, demonstrated that after twelve weeks of consuming a 60% kcal high-fructose diet the expression of these same genes are no longer upregulated when compared to the control-fed mice (Figure 8). While there is a transient increase in gene expression of these fructolytic gene, it cannot be maintained for extended periods.

In this study, the expression of critical adipogenic genes were also examined after both 48-hours and twelve-weeks of dietary intervention in the FVB/N mice. The expression of PPARγ, ACC, ACLY, ACSS2, and FASN were all assessed for the critical roles they play in adipogenesis and lipid storage (Figure 9). While these mice had no significant difference in body weight between the control and fructose-fed animals, there was a significant increase in epididymal adipocyte size in the fructose-fed mice when accessed after twelve weeks of feeding (Figures 4 and 6). Many of the accessed lipogenic genes have previously been shown to be significantly increased after short-term high-fructose feeding [16, 66].

In this study, after 48-hours of high-fructose feeding, a significant increase in all measured adipogenic genes are observed when compared to their control-fed counterparts. The expression of ACLY and ACSS2 are most significantly upregulated in the 48-hour fructose-fed FVB/N mice as they generate acetyl-CoA which is used directly in lipogenic pathways [16]. These data illustrate how adipogenic pathways are quickly

upregulated after high-fructose feeding to store the excess triosephosphates created during the unregulated catabolism of fructose.

After twelve weeks of high fructose feeding, however, there is no significant difference these same adipogenic genes between the fructose-fed and control-fed mouse livers. This parallels the changes seen in fructolytic genes between the two time-points examined. Indeed, if less fructose is being fully catabolized in the liver there is less need for the increase in lipid storage.

Interestingly, at the same twelve-week timepoint, there are significant differences in these same adipogenic genes in the adipose tissue of the control and fructose-fed FVB/N mice (Figure 13). There was a significant increase in the expression of PPARγ in the adipose tissue of the fructose-fed mice when compared to their control-fed counterparts. As PPARγ is known to regulate the storage of fatty acids in adipose tissue this finding is expected [67]. It is well described how a fructose-enriched diet increases lipogenic genes in the liver, the production of VLDL, as well as serum triglyceride levels. With the increase in adipocytic PPARγ expression, the adipocytes can more readily store the increased serum triglycerides associated with the high-fructose diet. These data are in agreement with the increase in adipocyte size previously shown after twelve weeks of high-fructose feeding (Figure 5).

In contrast there is a significant decrease in the gene expression all measured adipogenic enzymes, including ACC, ACLY, ACSS2, and FASN. All of these genes are responsible for a different enzyme critical to the production of lipid. Due to the increased hepatic lipid production, the endogenous production of lipids in adipose tissue appears to decrease. The storage of lipid in a given adipocyte is not infinite so the significant increase

in serum triglycerides may already put adipocyte lipid storage at a maximum [68]. Further investigation into this phenotype is required to fully understand the adipogenic changes that are occurring after long-term high-fructose feeding.

The role of glucocorticoids was also accessed in this study. It has previously been suggested that glucocorticoid signaling can be effected in a liver actively metabolizing fructose [49, 55]. In our study, while there is a significant increase in 11β-HSD1, and the downstream glucocorticoid target gene SGK1, after 48-hours of high-fructose feeding, no significant differences are seen between the control and fructose-fed FVB/N mice after the twelve weeks on diet (Figure 12).

The expression and production of FGF-21 was investigated in this study as both fructose metabolism and glucocorticoid signaling have been shown to influence the production of the hepatokine [64, 69]. FGF-21 is known to be highly influential maintaining metabolic homeostasis in stressful situations, such as prolonged fasting or high-fructose feeding [61, 70, 71]. FGF-21 is known to induce numerous positive health outcomes in humans including improve insulin sensitivity, reducing hepatic steatosis, and even improving cognition [72-74]. FGF-21 drug trials have been conducted in obese patients; however, circulating FGF-21 is already elevated in obese individuals and are thought to be resistant to the benefits of FGF-21 [63]. Although the long-term effects of obesity on the production of FGF-21 have been thoroughly investigated, the impact of continued fructolysis on the regulation of FGF-21 is yet to be fully elucidated.

To better understand the mechanism by which fructose modulates FGF-21, this study investigated the production of FGF-21 and the expression related genes. After 48-hours of high-fructose feeding in FVB/N there is a significant six-fold increase in the

expression of hepatic FGF-21 when compared to the control-fed mice, and further significant increases to both its co-receptor and receptor – βKlotho and FGFr1, respectively. After twelve weeks of high-fructose feeding, however, there is no significant difference in FGF-21 or related genes when compared to the control-fed mice (Figure 10). There is even a trend towards the decreased expression in FGF-21 after twelve weeks of high-fructose feeding.

Furthermore, serum levels of FGF-21 were assessed in both the 48-hour and twelve-week arms of the study (Figure 11). In concert with the trends displayed in the gene expression results, there is a significant increase in the serum levels of FGF-21 after 48-hours of high-fructose feeding, but there is a significant decline in serum FGF-21 after twelve weeks of high-fructose feeding after comparing these levels to the control-fed FVB/N mice. These data are similar to most of the results analyzed between the 48-hour and twelve week studies, where the fructolytic and adipogenic genes are all increased after 48-hours, but after continued high-fructose feeding for three months no significant difference in signaling is seen between the control and fructose-fed cohorts. As FGF-21 is known to exert numerous positive health effects, the decline to FGF-21 over extended periods of high-fructose feeding may contribute to the development of metabolic disease associated with a high-fructose diet.

The increases to the fructolytic, adipogenic, and FGF-21-related genes were all expected after 48-hours of high-fructose feeding due to previous studies that have primarily focused on short-term dietary studies. In this study, for the first time the differences between short-term and long-term consumption of fructose were assessed in FVB/N mice. This mouse model allowed for the assessment of metabolic changes due to

high-fructose feeding without the confounding impacts of obesity and insulin-resistant which exudes metabolic differences by themselves.

In the long-term, twelve-week assessment of high-fructose feeding on FVB/N mice this study found that there are no longer significant increases in fructolytic, adipogenic, or FGF-21-related genes. One interpretation of this is that over such an extended period of high-fructose feeding the small intestine adapts to compensate for such high-levels of fructose. It is well established, at least at low levels of fructose consumption, that the small intestine is able to catabolize the consumed fructose into glucose, acetate, and other byproducts which are easily metabolized by the liver, and protects the liver from the deleterious effects of hepatic fructolysis.

Moreover, it has also been demonstrated that the gut can adapt overtime to metabolize more fructose if fructose is consistently consumed [16, 17]. In addition to the upregulation of fructolytic enzymes in the gut, the changing microbiome can impact gut metabolism as well [75]. Recently it has been firmly demonstrated that a fructose-enriched diet can alter the microbiome, which potentially alters the amount of fructose metabolized by the liver [76-78]. As this current study did not investigate the gut metabolism of fructose, more work is required to determine if it can compensate to catabolize a 60% kcal high-fructose diet.

As the small intestine has never been shown to metabolize that amount of fructose, another method of blunted fructolysis must occur to instigate the downregulation of the fructolytic, adipogenic, and FGF-21-related genes. As previously noted, the hepatic metabolism of fructose is an expensive process that requires a lot of ATP, as well as other biological cofactors, to complete the catabolic process. For example the initial

phosphorylation step of fructolysis, catalyzed by KHK, requires ATP and it has been reported that fructose-1-phosphate often builds in hepatocytes after high-fructose feeding, and ATP stores can begin to become depleted [21, 31]. If cellular stores of ATP, as well as other biological cofactors continue to become depleted, it is not unrealistic to hypothesize that the normal hepatic fructolysis would not be able to continue.

While not considered the canonical fructolytic pathway, hepatic fructose can be converted directly into fructose-6-phosphate by hexokinase and inserted higher into the glycolytic pathway. Furthermore, a separate isoform of KHK is expressed in a variety of tissues which theoretically allows for the extrahepatic metabolism of fructose. If non-canonical version of fructolysis became heightened when the standard pathway could no longer cope, the increase in fructolytic, and possibly adipogenic or FGF-21 related genes would no longer be upregulated as is seen in this twelve-week study. To fully understand how continuous high-levels of fructose alter hepatic metabolism future studies are required.

Throughout this study it has been shown that between short and long-term feeding drastic differences in the catabolism of fructose exist. While the fructolytic process is able to handle excess fructose in short bouts, long periods of high-fructose feeding are not well handled by standard hepatic metabolism. As an increase in fructose consumption is associated with numerous negative health outcomes, it is imperative to better understand how these changes to hepatic fructolysis over time may contribute to the formation of disease.

Chapter IV

Additional Studies and Future Research Directions

A. Introduction

Although great strides have been made to understand the negative health outcomes associated with a high-fructose diet, more work is still required to fully understand the distinct metabolism of fructose and how this causes metabolic disease. Throughout the studies presented in this thesis, the effects of high-fructose feeding were investigated in FVB/N mice. As shown in chapter II, these mice are resistant to fructose-induced obesity and insulin resistance, which allows for a thorough investigation of fructose metabolism without the confounding factors of metabolic disease which are known to exert their own metabolic effects.

Although the fructose-fed mice exhibited no signs of weight gain, significant differences were seen between the fructose and control fed mice in peripheral tissues. For example, differences were shown in both adipocyte size and BMD, were the fructose-fed mice had a significant increase in both after twelve weeks of high-fructose feeding. Although these differences were seen in peripheral tissues after a long-term fructose rich diet, the central hepatic metabolism of fructose was no longer increased at the same time-point.

To fully understand why these differences in fructose metabolism occur over time, and how it may relate to the development of metabolic disease associated with high-

fructose feeding, further studies are required. In this final chapter, future studies will be outlined that answer some of these remaining questions about the metabolism of fructose.

B. Tracer Studies

As outlined in chapter III, much work has been done to understand the short-term effects of high-fructose feeding, yet less studies have been conducted to understand the long-term metabolic changes associated with a high-fructose diet, in what ways they may differ from acute fructose related metabolism, and how this may be implicated in the progression of multiple diseases. Almost all tracer studies complete to date are conducted after a single fructose challenge. While these past studies are essential to understanding the standard metabolism of fructose, it has been shown here how many of these pathways are altered after a prolonged high-fructose diet. Conducting tracer studies throughout a time-course of high-fructose feeding over weeks and months will be essential to showing how hepatic fructolysis is altered with a continuous high-fructose diet, and may shed light to why dietary fructose is associated with such an increase in metabolic disease.

To conduct this study, fructose labelled with isotopic carbon will be ingested by the FVB/N mice via oral gavage at multiple points before and after dietary intervention. Mice separated into two cohorts - a fructose-fed group and a control-fed group – will undergo the tracer protocol before dietary intervention at four weeks of age, two days after the start of their respective diets, followed by further studies at two weeks, four weeks, and twelve weeks. Throughout the entirety of the study, the mice will be fed the same control and high-fructose diets outline in the above chapters (Figure 1).

From these studies, the fate of specific carbons in the fructose molecule can be determined as where they end up in the metabolic pathway. This data will enable a proper analysis of how the metabolism of fructose changes over time with a prolonged diet. As the fate of the fructose carbons are hypothesized to change after weeks and months on diet, the effects of these metabolic changes on the development of metabolic disease could be more precisely predicted. Upon the completion of this study a more comprehensive knowledge of fructose metabolism will be obtained, especially as it relates to long-term high-fructose feeding.

C. Mitochondrial Bioenergetics

In the previously outlined study utilizing tracer studies, the different metabolic fates of fructose will be investigated over a long-term high-fructose diet. Although that study is imperative for a full understanding of how the catabolism of fructose is altered over a time-course of high-fructose feeding, it cannot by itself probe for the source of the potentially altered metabolic pathway. To examine the basis for the altered metabolic pathways after long-term high-fructose feeding, I believe that an investigation into the bioenergetics of hepatic fructose metabolism is required.

Under conditions of continued hepatic fructolysis, organic cofactors such as ATP are clearly being depleted, yet the metabolic implications of this are not clearly defined [21]. Oxidative stress and mitochondrial dysfunction are clear markers of fructolysis, yet more work is needed to understand how a high-fructose diet clearly affects bioenergetic functions. Completing respiratory profiles from mitochondrial isolated from hepatic tissue

after different lengths of high-fructose feeding will shed light into hepatic adaption to high-fructose feeding and how this leads to disease progression.

A mitochondrial respiratory analysis can be conducted in parallel with the previously proposed study regarding the isotopic tracing of fructose. Similarly, FVB/N will be fed a control or fructose-rich diet for a maximum of twelve weeks, where separate cohorts will be sacrificed at all of the timepoints mentioned in the previously designed study: Prior to diet, two days after dietary intervention, two weeks, four weeks, and twelve weeks. At each time of sacrifice mitochondria can be isolated via centrifugation protocols so that a respiratory analysis can be conducted at each timepoint.

As mitochondrial dysfunction has been noted after short bouts of high-fructose feeding, I hypothesize that the respiratory profiles of the hepatic isolated mitochondria from the fructose-fed animals will continue to decline as the diet is extended [79]. The hypothesized drop in oxidative capacity, and subsequent stores of ATP would limit the potential for fructose to be catabolized through its canonical pathways which are energy intensive and completely unregulated. Alternative pathways of fructolysis may prevail such as through hexokinase, which would insert fructose byproducts further up in the glycolytic pathway, or potentially through the extrahepatic metabolism of fructose via alternative isoforms of KHK which are present in peripheral tissues.

From this study a much better understanding of how mitochondrial function changes over a time-course of high-fructose feeding will be acquired. While previous work has shown a decline in bioenergetic capacity after high-fructose feeding, the addition of a time-course will give a much greater breadth to our current understanding of how a long-term high-fructose diet alters hepatic metabolism. Furthermore, at the completion of

this study a much more extensive knowledge of high-fructose diet induced bioenergetic would be obtained in an effort to understand the molecular basis of the altered fructolytic pathway outlined in the previously proposed study.

D. Conclusions

The consumption of fructose and the development of metabolic disease continue to increase in parallel throughout the world. As we continue to gain a more thorough understanding of how fructose is metabolized and its deleterious effects on the development of multiple metabolic disease, we will also be able to better treat and prevent many of these diseases which are increasing in incidence throughout the world. For this reason, it is imperative that continued investigations into the metabolic outcomes of fructose consumption are conducted.

Through these chapters, we sought to develop on previous efforts to understand how long-term high-fructose feeding effects multiple metabolic pathways and predict how these changes may impact the development of metabolic disease. Through the outlined studies, the effects of high-fructose feeding on FVB/N mice have been profiled, while paying attention to how differential lengths of high-fructose feeding alters hepatic fructolysis and related metabolic pathways. It is clear that a continued high-fructose diet not only alters hepatic and peripheral metabolic pathways, but that these changes are associated with the development of metabolic disease.

With future studies – some of which are outlined in this chapter – it will be possible to fully understand the metabolic pathway of fructolysis, how it is altered with continual

high-fructose feeding, and how these changes cause the development of metabolic disease. When conducted in the future, these studies and others will complete our knowledge of the metabolism of fructose, especially with how it relates to the development of metabolic disease. With this knowledge it may be possible to not only create a better treatment for fructose-induced metabolic dysfunction, but also generate a more complex solution to how people and food developers limit the incorporation of high-fructose corn syrup into so many foods consumed by people throughout the world.

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