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**A high fructose diet alters affective-like behavior and metrics of synaptic mitochondrial function differentially in male and female rats**

A thesis in partial fulfillment of the requirements for the degree of Master of Science in Anatomy and Neurobiology at Virginia Commonwealth University

By

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## Abstract

### A HIGH FRUCTOSE DIET ALTERS AFFECTIVE-LIKE BEHAVIOR AND METRICS OF SYNAPTIC MITOCHONDRIAL FUNCTION DIFFERENTIALLY IN MALE AND FEMALE RATS

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A thesis in partial fulfillment of the requirements for the degree of Master of Science in Anatomy and Neurobiology at Virginia Commonwealth University

Virginia Commonwealth University, 2019

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Fructose consumption has become a normalized part of the standard American diet over the past 40 years. Adolescents are the greatest consumers of fructose, consuming as much as 21% of their daily energy intake in added sugars, likely attributed to the introduction of sweetened beverages. Adolescence is a critical time point in development, in particular due to the far-reaching actions of the hypothalamic-pituitary-adrenal axis, which matures during this developmental phase. While fructose consumption is a known risk factor of metabolic syndrome, type 2 diabetes, and cardiovascular disease, there is increasing evidence that fructose consumption influences brain and behavior. Recently, more interest has been focused on mitochondrial dysfunction as a potential link between metabolic stress and modifications of the central nervous system. Mitochondria control energy metabolism and cellular signaling, placing them in the unique position of both regulating and being vulnerable to alterations in energy homeostasis. Sex-differences are well categorized in the presentation of metabolic symptoms associated with excessive fructose consumption. Thus, it is important to characterize sex-specific outcomes in the arena of brain and behavior in order to develop better strategies for mitigating the effects of fructose consumption. Therefore, I determined the extent to which a high fructose diet modified physiological outcomes, serum corticosterone, and affective-like behavior in male and female rats. In addition, I examined the potential of excessive fructose consumption to modify synaptic mitochondrial respiration at baseline and following an acute stress experience. In male rats, weight, caloric efficiency, and circulating blood glucose was unaffected by fructose consumption. Serum corticosterone was increased following an acute stress event, and this increase was modified by diet. In addition, fructose consumption resulted in decreased affective-like behavior in the open field test and synaptic mitochondrial respiration



was altered by both diet and acute stress experience. In females, excessive fructose consumption altered weight and caloric efficiency, but not circulating blood glucose. Females demonstrated increased depressive-like behavior in a forced swim test. Corticosterone concentrations were increased by acute stress experience, but not by diet, and synaptic mitochondrial function was only modified by diet in groups that underwent an acute stressor.

In this thesis, I will describe our current knowledge of fructose metabolism and associated pathophysiological outcomes. This is followed by a description of the experimental model system, which was used to demonstrate the effect of the dietary challenge on behavioral outcomes and synaptic mitochondrial respiration in male and female rats. I will then describe the results collected and discuss the ramifications of these results and the potential underlying mechanisms to account for differences. The results of this study should help us to better understand the role that dietary challenges in adolescence play in behavioral disruptions in adulthood and how mitochondrial function may serve as a link between metabolic stress and behavioral responses.

## Chapter One

### Introduction

In the past 40 years, fructose consumption has become a normalized part of the standard American diet due largely to the invention and introduction of added caloric sweeteners such as high fructose corn syrup into commercial food products (Marriott et al., 2009). As of the early 2000's, added sugars accounted for approximately 16% of all caloric intake, as demonstrated by a nationally representative survey conducted from 1994 to 1996 (Bray et al., 2004). Below I review data related to the contributions of fructose to metabolic syndrome, which may be driven by the way fructose is metabolized. Although metabolic syndrome is a well-appreciated consequence of a diet high in fructose, emerging evidence also suggests that fructose can impact brain and behavior (Harrell et al., 2015). I hypothesized that fructose consumption beginning at weaning would result in metabolic disruptions accompanied by increases in anxiety-like and depressive-like behavior in both male and female rats. Further, I hypothesized that these alterations in affective-like behavior would be paralleled by decreases in synaptic mitochondrial respiration, and that alterations in respiration would be modified by sex. Collectively, an understanding of the impact of fructose on brain and behavior will build a framework within which solutions to fructose-altered

metabolic and behavioral patterns can be ascertained. In addition, these data will raise awareness regarding the long-term implications of fructose as a dietary choice.

### ***Fructose Metabolism***

High fructose corn syrup was first introduced in the 1970s and is still the dominant caloric sweetener consumed in the American diet. High fructose corn syrup was first developed using a glucose isomerase to convert cornstarch to glucose and then further into fructose (Marshall et al., 1957). This produced an inexpensive alternative to sucrose and other simple sugars already present in the American diet, and generated new opportunities for food and beverage manufacturers around the world. High fructose corn syrup has since become a favorite additive in sweetened non-juice beverages, soft drinks, dairy products, baked goods, canned fruits and candies (Bray et al., 2004). While the benefits of added sugars such as fructose were clear for manufacturers of these goods, the implications of a shift in energy availability on the general consumer population are still subject to research.

The body utilizes glucose as its primary source of fuel, but when confronted with changes in energetic availability such as an overabundance of fructose, a state of disarray can arise. This is because the metabolism of glucose and fructose differ in a number of ways that have profound implications for energetic homeostasis. At the molecular level, fructose and glucose possess the same molecular formula,  $C_6H_{12}O_6$ , but differ in chemical groups present, with glucose possessing an aldehyde group on the carbon chain and fructose

possessing a ketone group. Glucose and fructose are absorbed at different places in the intestinal tract, with glucose being absorbed higher up in the small intestine (Havel, 2005). Upon ingestion, fructose is absorbed by the intestinal epithelium and transported into the hepatic portal vein. Fructose is preferentially metabolized by the liver, where specific enzymes are present for the metabolism of fructose (Mayes, 1993), such that little fructose manages to escape the liver and enter systemic circulation (Havel, 2005). Studies of tube feeding fed rats and starved rats showed a fractional uptake of 55% and 71%, respectively, of fructose by the liver (Topping & Mayes, 1971). In humans, it was shown that the liver metabolized at least half of intravenously administered fructose (Mendeloff & Weichselbaum, 1953). Once in the liver, ATP rapidly phosphorylates fructose to fructose-1-phosphate, catalyzed by the first enzyme in fructose pathway, fructose kinase (Hers, 1952). Furthermore, fructose bypasses the main rate controlling stage of glycolysis catalyzed by phosphofructokinase (Underwood & Newsholme, 1965). Without this rate-limiting step substrate availability to metabolic pathways is substantially increased, with the main byproducts of fructose consumption being glucose, glycogen, lactate, and lipids (Exton & Park, 1967).

Fructose metabolism also has implications for endocrine system modification. Unlike glucose, fructose absorbance does not stimulate the release of insulin from pancreatic beta cells (Tappy & Le, 2010). Other essential metabolic hormones rely on insulin for regulation, such as ghrelin and leptin. Leptin, the “satiety hormone” rises in response to insulin, and ghrelin, the “hunger hormone” decreases in response to insulin (Teff et al., 2004). As fructose does

not stimulate the same insulin response as glucose, leptin and ghrelin levels are reduced and increased, respectively upon the ingestion of fructose. This has the potential to result in excessive caloric intake, which contributes to obesity and other metabolic disorders. Additionally, leptin acts on the liver to encourage fat oxidation and mobilization. Fructose-induced leptin resistance is suggested to be a mechanism in non-alcoholic fatty liver disease (NAFLD), a disease that is increasingly associated with metabolic syndrome (Roglans et al., 2007; Vila et al., 2008; Paschos & Paletas, 2009).

While excessive fructose consumption encourages caloric intake increases, fructose metabolism initiates qualitative changes to energy metabolism due to the differences from glucose metabolism described above. Experimental studies have linked disorders such as fatty liver and metabolic syndrome to fructose consumption independent of excessive calorie intake (Nakagawa et al., 2006; Roncal-Jimenez et al., 2011; Gersch et al., 2007). This further supports the idea that the differential metabolism of fructose, and not only its ability to entice excessive energy intake, contributes to pathophysiological outcomes.

### ***Metabolic Syndrome and Fructose***

In correlation with the increase in fructose consumption in recent years, incidence of metabolic syndrome has been steadily on the rise (Ferder et al., 2010). Metabolic syndrome is defined as a clustering of several known physiological risk factors, including, but not limited to, insulin resistance,

prothrombosis, hypertension, obesity, and dyslipidemia (Eckel et al., 2005). Metabolic syndrome is used to identify patients at a particularly high risk for cardiovascular disease and type 2 diabetes (Zimmet et al., 2001). In addition, many of the risk factors attributed to metabolic syndrome possess similar underlying pathophysiological mechanisms. Fructose consumption stimulates metabolic disruption and has been linked to insulin resistance, hypertension, diabetes, and obesity. In a study of male rats that were fed a diet enriched with fructose initiated in adolescence, fructose-fed rats presented with dyslipidemia, insulin resistance, hypertension, and early signs of liver malfunction via higher liver weights, all of which are components indicative of metabolic syndrome (Dupas et al., 2017). Other studies have implicated fructose consumption in the development of metabolic syndrome, and consumption of a high fructose diet (HFD) has been used to induce animal models of metabolic syndrome in rats and mice (Hwang et al., 1987; Ishimoto et al., 2012). In one study, male Sprague-Dawley rats were fed either a control diet, a 60% fructose diet, or fructose administration via a 10% solution added to drinking water for 8 weeks. Both the 10% group and the 60% group experienced hypertension, hyperuricemia, and hypertriglyceridemia. Interestingly, there was a step-wise progression in elevations, with the 60% consumption group having higher levels than the 10% group. Additionally, the group consuming the 60% diet experienced renal disturbances in the form of renal hypertrophy, cortical vasoconstriction, calciphylaxis, and glomerular hypertension (Sanchez-Lozada et al., 2007). In hamsters, chronic fructose feeding resulted in elevated plasma triglycerides from

intestinal *de novo* lipogenesis and increased lipoprotein production (Haidari et al., 2002; Lewis et al., 2005). In hepatocytes, *de novo* lipogenesis results in the conversion of fructose into fatty acids. This conversion to fatty acids was demonstrated *in vivo* in rats (Bar-On & Stein, 1968) and in isolated hepatocytes (Topping & Mayes, 1972). In humans, increased fructose consumption stimulated lipogenesis, resulting in dyslipidemia and hepatic and adipose tissue insulin resistance (Faeh et al., 2005).

### ***Adolescents and Fructose Consumption***

Of the population consuming fructose, adolescents consume the largest proportion of fructose, consuming as much as 21.4% of their total energy intake in added sugars (Welsh et al., 2011). The majority of added sugar consumption comes from sweetened beverages such as soft drinks and fruit juice, and children are the highest consumers of these beverages (Wang et al., 2008). The consumption of added sugars in children is positively correlated with a number of negative outcomes in both adolescence and further on into adulthood. In the United States, adolescents have been experiencing higher incidence of metabolic syndrome, obesity and type 2 diabetes with the rise in consumption of calorically sweetened beverages and foods (Vatarian et al., 2007). As of 2017, 1 in 3 children are classified as overweight or obese (Kumar & Kelly, 2017). The prevalence of childhood obesity is associated with the emergence of disorders, such as type 2 diabetes, dyslipidemia, hypertension, and other diseases previously considered to only emerge in adulthood (Welsh et al., 2011).

Furthermore, the consumption of a HFD in childhood has been directly linked to risk factors associated with cardiovascular disease (Morrison et al., 2009; Welsh et al., 2011).

Aside from the emergence of pathophysiology in adolescence, excessive fructose consumption has been shown to have lasting implications into adulthood. Childhood adiposity has been shown to track into adulthood (Serdula et al., 1993; Singh et al., 2008). Furthermore, studies have shown that adolescent body mass index (BMI) is positively associated with a number of metabolic risk factors in adulthood such as development of metabolic syndrome, insulin resistance, type 2 diabetes, and coronary heart disease (Srinivasan et al., 2002; Morrison et al., 2008; Baker, 2007). In adults, increased consumption of fructose is associated with an increased risk of diabetes and metabolic syndrome (Montonen et al., 2007; Bazzano et al., 2008; Dhingra, 2007). Broadly put, this shift in dietary energy homeostasis brought on by overconsumption of added sugars, such as fructose, is correlated with increased incidence of peripheral metabolic pathophysiology in childhood and adulthood.

### ***Neural and Behavioral Consequences of Fructose***

It is well established that a HFD negatively impacts peripheral metabolism and contributes to pathophysiological outcomes such as metabolic syndrome. However, the neurological implications of a HFD are less understood. As with the periphery, glucose is traditionally considered the main source of fuel for the brain (Harris et al., 2012; Mergenthaler et al., 2013). It is already known that hepatic



fructose metabolism utilizes a different set of enzymes that allows it to bypass the first rate-limiting steps of glycolysis. Likewise, similar enzymes are present in areas of the central nervous system (CNS) that play a part in energy balance regulation (Funari et al., 2005; Shu et al., 2006). Furthermore, the ketonic nature of fructose necessitates different absorptive and metabolic pathways in the brain, as it does in the gastrointestinal system. While over 60% of fructose is metabolized by the liver and transformed into lipids, lactate, glucose, glycerol, and glycogen, the remaining fructose is available for extrahepatic metabolism and reuptake into tissues (Sun & Empie, 2012).

The direct impact of fructose on cerebral metabolism is less understood. Fructose is passively transported across membranes by a glucose transporter, GLUT5. While there are other members of the GLUT family, GLUT5 is the only transporter specific for fructose, and is unable to transport glucose or galactose. Other members of the GLUT family possess varying degrees of fructose sensitivity. GLUT5 has been identified in different tissues within the brain, including mouse cerebellum (Funari et al., 2005), rat hippocampus (Shu et al., 2006), human microglia (Payne et al., 1997), and human blood brain barrier (Mantych et al., 1993). Furthermore, cerebral glucose transporters, including GLUT5, has been shown to be differentially expressed based on brain region, age and sex (Kelly et al., 2014). The expression of GLUT5 and of enzymes necessary for fructose metabolism indicates that the brain is able to utilize fructose as an energy source. Nevertheless, the utilization of fructose and role of GLUT5 within the brain is still uncertain. In rodents, central injection of fructose

led to a decrease in hypothalamic ATP levels and corresponding increase in AMPK activation, whereas glucose resulted in a rapid increase in ATP levels within the same time frame. The same study also reported a subsequent drop in hypothalamic malonyl-CoA upon central fructose injection, an important component in feeding behavior signaling (Cha et al., 2008; Wolfgang, 2007). In humans, functional Magnetic Resonance Imaging (fMRI) was used to study the effects of glucose and fructose ingestion on regional cerebral blood flow (CBF), an indirect indication of neural activation. Following ingestion of a glucose bolus, there was a significantly greater reduction in hypothalamic CBF, compared to hypothalamic CBF following the ingestion of a fructose bolus (Page et al., 2013). Furthermore, glucose ingestion stimulated increased activity between the hypothalamus, thalamus, and striatum whereas fructose ingestion only resulted in increased activity between the hypothalamus and thalamus (Page et al., 2013). Collectively, these findings suggest that excessive consumption of fructose is able to modify cerebral metabolism on multiple levels.

Because the CNS, particularly the hypothalamus, is the primary regulator of energetic homeostasis for the human body (Morton et al., 2006; Elmquist et al., 2005; Meister, 2007; Myers et al., 2008), perturbations to energetic balance have the potential to initiate neurological consequences, outside of the traditional scope of metabolism. Previous studies in male rats have shown that a high fructose diet initiated in adolescence alters the Hypothalamic Adrenal Pituitary (HPA) axis transcriptome, metabolic outcomes, and results in increased depressive-like behavior in male rats (Harrell et al., 2015). Metabolic disruptions

and associated etiology have consistently been associated with increased risk of affective-like disorders, and the bidirectional relationship between dysregulated metabolism and mood disorders is the subject of increased research (McIntyre et al., 2011; Musselman et al., 1998; Perlmutter et al., 2000). In clinical studies, metabolic syndrome and diabetes are highly co-morbid with depression and anxiety. In a study of both men and women, participants with any of 5 obesity-related comorbidities were found to have significantly higher incidence of current depression, lifetime diagnosed depression, and lifetime diagnosed anxiety (Zhao et al., 2009). In another study of both males and females, metabolic syndrome was significantly associated with having a current anxiety disorder and lifetime major depression (Kahl et al., 2015). Another study of 1598 subjects, both male and female, found that metabolic syndrome occurrence was associated with an increased prevalence of depression, but not anxiety, irrespective of gender and overweight/obesity status (Skilton et al., 2007). Taken together, these studies demonstrate a clear association between metabolic dysfunction and incidence of mood disorders. Fructose may be a missing piece in understanding metabolic disruptions and their role in mental health, but further studies are necessary to understand this connection.

### ***Metabolism and Mitochondria***

Given that a HFD alters peripheral metabolism, and alterations in affective-like behavior have previously been demonstrated in male rats it raises the question- to what extent is brain metabolism affected by a fructose-induced

change in energy availability? At the cellular level, mitochondrial function is critical to examine when investigating the neural underpinnings of behavioral alterations in relation to global modifications in energy homeostasis. Mitochondria are present in the cytoplasm of all mammalian cells, including neurons and are responsible for transforming energetic substrates and oxygen into ATP that can be used for energy dependent reactions. Mitochondria are also responsible for other critical functions such as cellular calcium buffering, reactive oxygen species (ROS) production, and antioxidant mechanisms. Mitochondria are particularly sensitive to the metabolic state of an organism. The oversupply of energetic substrates relative to demand has been shown to have an adverse effect on mitochondrial structure and function, including increased fragmentation (fission), increased production of ROS, and mtDNA damage (Picard & Turnbull, 2013). The metabolic machinery of mitochondria produces ROS when single electron species are passed down the electron transport chain to terminal oxygen during ATP production. At low levels, ROS are necessary for cellular function and play important roles in cellular differentiation, apoptosis, immunity, and intracellular signaling (Ghosh, 1998; Tohyama & Yamamura, 2004; Roberts & Sindhu, 2009; Lambeth, 2004; Lambert & Brand, 2009; Balaban et al., 2005). Importantly, a state of oxidative stress arises when mitochondrial production of ROS outpaces its ability to inactivate ROS through antioxidant machinery, which leads to damage of biomolecules and mtDNA (Yakes & VanHouten, 1997).

In instances of metabolic pathophysiology such as diabetes, the effects on oxidative stress, damage to mtDNA, and altered mitochondrial function are well

characterized (Suzuki et al., 1999; Giugliano et al., 1996; Baynes, 1991). Furthermore, levels of oxidative stress are increased in patients with metabolic syndrome (Ford et al., 2003; Armutcu et al., 2008). Given the correlation between HFD and pathophysiological disorders such as metabolic syndrome and diabetes, it is pertinent to investigate the effects of fructose on mitochondrial function, especially in the context of behavioral alterations and cerebral metabolism. Recently, advances in isolation of intact nerve terminals and *in vitro* respirational determination made it possible to investigate cerebral metabolism in animals at the time of euthanasia. Synaptosomes are intact nerve terminals formed by shearing forces during homogenization of neuronal tissue (Gray & Whittaker, 1962). The resealing of the plasma membrane following homogenization generates a “miniature cell” with intact synaptic vesicles and mitochondria in cytoplasm, so that mitochondrial bioenergetics can be studied using real-time analysis (Nicholls et al., 2003). Synapses are the energetically demanding area of neuronal communication, and therefore ideal areas to investigate the quality of cerebral metabolism, specifically mitochondrial integrity.

### ***Importance of Studying Sex as a Biological Variable***

While there is already a great deal of research on how a HFD affects an organism on many levels, it does not give the full picture. The majority of previous studies have been conducted in only male subjects; however, females are equally susceptible as males to dietary perturbations and associated conditions such as metabolic syndrome, Type 2 diabetes, and obesity. The

beginning of puberty marks the endocrine system's regulation of diet-induced effects including insulin sensitivity, hypertension, and lipid levels (Vasudevan et al., 2005; Chen et al., 1992; Galipeau et al., 2002; Louet et al., 2004) and at this point sex differences in metabolism are magnified. Interestingly, women are twice as likely to experience depression and anxiety in their lifetime as men, and this trend emerges after puberty and persists for the next 30-40 years (Ford & Erlinger, 2004; Cyranowski et al., 2000). Despite this interesting co-occurrence, little is known about differences in neural mitochondrial function between the sexes. Mitochondrial dysfunction is also implicated in a large number of these pathologies, including cardiovascular and neurodegenerative disorders (Duchen & Szabadkai, 2010; Demarest & McCarthy, 2015). Mitochondria display prominent sex-specific and tissue specific behavior in pathophysiological states (Ventura-Clapier et al., 2017). In mice, studies show that young female mice have lower oxidative stress and a higher reduced NADH-linked respiration rate when compared with young males and aged females. Further analysis of brain steroid levels revealed higher pregnanolone and progesterone brain levels in young females, which decreased with aging in females and were lower in males, suggesting these steroids' contribution to sex-dependent changes in brain mitochondrial function (Gaignard et al., 2015). In a study analyzing liver mitochondria, and synaptic and non-synaptic brain mitochondria of rats, Borrás et al. found that peroxide production is significantly decreased in females compared to males. Additionally, males demonstrated higher levels of oxidative damage to

mitochondrial DNA, and females had higher glutathione levels than males across all mitochondrial types (Borras et al., 2003).

There is prominent evidence on sex as a biological variable in metabolic disorders, mitochondrial dysfunction, and clinical presentation of anxiety and depressive disorders. Given that previous studies have demonstrated the effect of a HFD on affective-like behavior in male rats it is necessary to include females in this study in order to better elucidate the effects of excessive fructose consumption on affective-like behaviors and mitochondrial dysfunction in adulthood.

I hypothesized that fructose consumption beginning at weaning would result in metabolic disruptions accompanied by increases in anxiety-like and depressive-like behavior in both male and female rats. Further, we hypothesized that these alterations in affective-like behavior would be paralleled by decreases in synaptic mitochondrial respiration, and that alterations in respiration would be modified by sex. Following a more in depth description of the materials and methods utilized in this study, I will outline the data collected in my study and then discuss the implications of the results.

## Chapter Two

### Materials and Methods

#### *Animal Husbandry*

Timed pregnant Wistar rats (n=6) were procured from Charles River (Morrisville, N.C.). All animals were housed in a temperature (20-23°C) and humidity (60%) controlled colony room in static cages. The room was kept on a 14:10 light:dark cycle. Litters were culled on post natal day (PND) 3 to eight pups per litter (male = 4 and female = 4). This was done to ensure an equal sample size of males and females with exception of one timed pregnant Wistar rat, which produced a litter with only one female pup. The pups were weaned on PND 22 (n= 46) and pair housed with same-sex, non-sibling cage mates for the duration of the study.

Cage mates were assigned to either a control chow diet (Males n=12; Females n=10) or a high fructose diet (Males n=14; Females n=10) on PND 25. All studies were conducted in accordance with Institutional Animal Care and Use Committee of Virginia Commonwealth University and National Institutes of Health Guide for the Care and Use of Laboratory Animals.



### ***Diet and Metabolic Measurement***

Animals were either maintained on a standard chow diet or placed on a high fructose diet beginning at PND 25 and remained on the assigned diet until their experimental end point. The chow diet was comprised of the Envigo Lab Diet 7012 (Teklad LM-485), while the High fructose diet (HFD) was 10% kcal fat and 55% kcal fructose (Research Diets D050111802), and both diets contained 19% kcal protein. Total food consumption was tracked through the duration of the experiment by weighing the total remaining food in the cage twice weekly before the food was replaced with a pre-weighed amount of new food. Attention was paid to food remaining on bottom of cage and all rats received water *ad libitum*. Weights for each animal were taken and recorded biweekly and an average weekly weight was calculated. Utilizing average weekly weight coupled with weekly food consumption, caloric efficiency (milligram weight gained per kilocalorie consumed) was calculated. Due to rats being pair housed, this measure is imprecise but serves to elucidate metabolic consequences as a result of the experimental diet.

### ***Blood Glucose Testing***

Blood glucose levels were obtained from experimental subjects at 3 times over the course of their lives: end of adolescence (PND 57), end of week 10 on assigned diet (prior to behavioral testing), and end of dietary week 12 (prior to

experimental endpoint). To accomplish this, a tail prick was done in the lateral tail vein using a sterile 25 gauge needle. A Freestyle Glucometer with Freestyle Lite test strips (Abbott Diabetes Care Inc., Alameda, CA) was used to obtain the reading.

### ***Vaginal Cytology***

Vaginal cytology was used to assess estrus cycle stage in the female rats over an 8-day period from PND 75 to PND 83 prior to behavioral testing. Rats were restrained by wrapping them in a bench pad while maintaining an exposed vaginal area. Using a 2 mL disposable pipette, approximately 2  $\mu$ L of phosphate buffer solution was used to lavage the vaginal cavity. The sample was smeared onto a slide and immediately imaged under a light microscope at 10x. Cycle stage was determined based on the relative amounts of either nucleated epithelial cells, neutrophils, or anucleated keratinized epithelial cells (Cora et al., 2015). On the same days that estrus cycle assessment occurred, males were similarly wrapped in a bench pad in order to control for any handling effect induced during the cytology procedure.

### ***Behavioral Testing***

Behavioral testing was conducted beginning after the subjects were on their designated diet for 10 full weeks at PND 95 and was conducted in order of

increasing anxiogenicity of the tasks. Behavioral testing consisted of a 10 minute open field test (PND 95), a 10 minute social interaction test (PND 96), and a 10 minute forced swim test (PND 116). All rats were habituated to the behavioral test suite for 3 days prior to testing. The open field test, social interaction test, and forced swim test were all conducted in the middle of the rats' light cycle. All tests were conducted at 100-200 lux. Behavioral testing was video recorded and tracked using Ethovision XT (Noldus Information Technologies; Leesburg, VA).

### *Open Field Test*

The open field test serves as a measurement of anxiety-like behavior (Prut & Belzung, 2003; Gould et al., 2009; Walsh & Cummins, 1976) and consists of exposing the test subject to an unfamiliar square arena for ten minutes. The rat was placed in the center of a novel, 75 x 75 cm square field with 35 cm high walls (Noldus) and allowed to freely explore for ten minutes. The subject was assessed on time spent in the center of the arena versus time spent in the periphery and for other measurements of anxiety-like behavior such as distance traveled, quantity of fecal boli, rearing and grooming behavior (Gould et al., 2009; Hall, CS, 1934).

### *Social Interaction Test*

A social interaction test was conducted the day following the initial open field test as a measure of anxiety-like and anhedonic behavior (File & Hyde, 1978; File & Seth, 2003). In this test the experimental animal was placed in the center of the same arena used for the open field test, now containing a novel stimulus rat, and allowed to explore and interact for 10 minutes. The stimulus rat was a younger, same sex, same strain animal and had prior exposure to the arena. The experimental subject's latency to interaction with the stimulus rat, number of approaches to the stimulus rat, and total time spent interacting with stimulus rat were scored by hand by a treatment-blind experimenter.

### *Forced Swim Test*

A forced swim test was used as a measurement of depressive-like behavior (Porsolt et al., 1978). The forced swim test is traditionally used to assess the efficacy of anxiolytic and antidepressant drugs over the course of two test sessions (Porsolt et al., 1978). In this case a single test was used to measure depressive-like behavior (Castro et al., 2010). The rat was placed into a circular, acrylic tank (diameter = 19cm; height = 46.5cm) filled with room temperature water deep enough so that the rat could not touch the bottom of the tank and swam for 10 minutes. Time spent inactive versus actively swimming, latency to inactivity, time spent struggling, and exhibition of coping mechanisms, such as

diving, were recorded. Inactivity was defined as the rat's limbs remaining motionless for at least 2 seconds and struggling was defined as the rat's head being above the water and limbs breaking the surface. Immediately following the forced swim test, rats were removed from the tank, placed in a cage on top of a heating pad, and allowed to rest for 20 minutes. Upon conclusion of the 20-minute rest period, rats were transferred into a separate room and rapidly decapitated. While the forced swim test is often used as an indicator of depressive-like behavior, the test also served as an acute stressor for the rats. In order to assess how an acute stressor impacted measures of mitochondrial function and endocrine system function, only half of the cohort underwent the forced swim test before euthanasia. The other half were euthanized without exposure to the test.

### ***Tissue Collection***

Following rapid decapitation trunk blood was collected and the kidney, spleen, uterus and portions of the liver were dissected, weighed, flash frozen and stored at -80°C. Brains were removed from subjects and bisected. The left hemisphere underwent rapid Golgi stain (analysis not included in this thesis document) and the right hemisphere was utilized for a cell mitochondrial stress test in the Seahorse XT Analyzer (Agilent).

### ***Corticosterone analyses***

Corticosterone was measured in serum in trunk blood collected at baseline and 30 minutes after the onset of an acute stressor, in the form of a 10 minute forced swim test, using a commercial ELISA (sensitivity 27 pg/mL, Enzo Life Sciences, Farmingdale, NY). All samples were run in duplicate with a CV<10%. For baseline samples, rats were transferred to a testing room to acclimate prior to decapitation. Rats were then transferred to a separate room and decapitated within two minutes of handling. Separation of the testing room and the room utilized for euthanasia ensured that transfer of scents and noise was hindered and that decapitation occurred before a rise in corticosterone. For the rats undergoing the forced swim test, the forced swim occurred in a separate room from that used for acclimation and for euthanasia. Following the ten minute forced swim test, rats were removed from the tank, briefly dried, and allowed to rest for 20 minutes on a heating pad in the same room where the swim occurred. Following the 20 minutes, rats were immediately decapitated in the same room where the baseline rats were decapitated. Following decapitation, trunk blood was collected and allowed to clot at room temperature before the clot was removed and remaining blood was placed on ice. Blood was centrifuged (Eppendorf 5810R) at 1800 rcf for 20 minutes at 4°C. Resulting serum was stored at -80°C for use in ELISAs.

### *Reagent Preparation*

In order to carry out the Corticosterone ELISA, manufacturer's instructions were used. Immediately before use, 1 ml of 1:100 Steroid Displacement Reagent (SDR) solution was prepared using deionized water. 10 µl of each sample was combined with 10 µl 1:100 SDR solution, vortexed, and allowed to sit for 5 minutes. After 5 minutes, 280 µl ELISA assay buffer was added to each sample and vortexed, generating a final sample dilution of 1:30. Next, the standard diluent (Assay Buffer 15) was prepared by diluting 10 ml of the supplied concentrate with 90 ml of deionized water. Wash Buffer was similarly made by diluting 5 ml of the supplied concentrate with 95 ml of deionized water. 8 Corticosterone Standards were prepared using 200,000 pg/ml Corticosterone standard solution warmed to room temperature and standard diluent (Assay Buffer 15). The concentration of corticosterone in standards 1 through 8 was 20 pg/ml, 12 pg/ml, 8 pg/ml, 4 pg/ml, 2 pg/ml, 0.5 pg/ml, 0.1 pg/ml, and 0.05 pg/ml respectively.

### *Assay Procedure*

100 µl of standard diluent was placed into the non-specific binding wells (NSB) and Bo wells, 100 µl of Standards 1 through 8 were placed in the appropriate wells and 100 µl of sample were pipetted into their designated wells. 50 µl of blue conjugate was then placed into each well, with exception of the Total Activity

(TA) and blank wells, and 50 µl of yellow antibody was placed into each well except the blank, TA, and NSB wells. The plate was incubated at room temperature on a plate shaker for 2 hours at approximately 500 rpm. Following incubation, each well was washed with 400 µl of wash buffer solution 3 times. 5 µl of blue conjugate was added to the TA wells and 200 µl of pNpp Substrate solution was added to each well. The plate was incubated at room temperature for 1 hour without shaking. After the final incubation, 50 µl of Stop solution was added to each well in order to stop the reaction and the plate was immediately read. The plate was read at 405 nm, with correction between 570 and 590nm, using a Biotek Synergy HTX Multimode Plate Reader (Winooski, VT).

### ***Cell Mitochondrial Stress Test***

#### *Preparation of Synaptosomes*

Synaptosomal isolation was adapted from Dunkley et al. (2008). Rats were euthanized via rapid decapitation and the whole brain was extracted. The cerebellum was removed, the brain was bisected, and the right hemisphere was placed in cold sucrose medium (320 mM Sucrose, 0.2 M EDTA, 5 mM Tris, pH 7.4) to remove excess blood. Tissue was homogenized in a 7mL Dounce glass homogenizer containing 4.5 mL cold homogenization buffer (320 mM Sucrose, 0.2 M EDTA, 50 mM dithiothreitol, 5.0 mM Tris, pH 7.4) by 5 and 6 strokes with the loose and tight plunger, respectively. The homogenate was centrifuged (Eppendorf 5810R) at 3600 rpm for 10 minutes at 4°C. The supernatant was



removed and 6 mL was layered on top of a discontinuous Percoll gradient (4 ml layers of 0,3,10,15,23 % Percoll in homogenization buffer) in a 26 mL centrifuge tube and spun at 32500g for 10 minutes at 4°C (JA-20 fixed angle rotor in a Beckman Avanti J-25 centrifuge). The synaptosomes were isolated from the band between the 15% and 23% Percoll layers, diluted in Ionic Media (20 mM HEPES, 10 mM D-Glucose, 1.2 mM Na<sub>2</sub>HPO<sub>4</sub>, 1 mM MgCl<sub>2</sub>, 5 mM NaHCO<sub>3</sub>, 5 mM KCl, 140 mM NaCl, pH 7.4), and centrifuged at 15000g for 35 minutes at 4°C (JA-20 fixed angle rotor in a Beckman Avanti J-25 centrifuge). The final synaptosome pellet was collected and protein concentration was determined (Nanodrop A280, ThermoFisher Scientific). Synaptosomal protein was resuspended in ionic media for respirometry (Choi et al., 2009).

### *Respiration Determination*

To quantify respiration, 40 µg of synaptosomal protein per well was aliquoted into a 24 well cell culture microplate (Agilent Technologies, Cedar Creek, MO) coated with Poly-D-Lysine. Plates were centrifuged at 3400g for 30 minutes at 4°C (Eppendorf 5810R centrifuge) in order to adhere the synaptosomes to the plate. The medium was then replaced with 500 µl of Seahorse XF assay media (Seahorse XF Base Medium (w/o Phenol Red), 10 mM Seahorse XF Glucose, 1 mM Seahorse XF Pyruvate, 2 mM Seahorse XF L-Glutamine). The microplate was then loaded into the Seahorse XFe24 extracellular flux analyzer according to the manufacturer's instructions. Wave Desktop 2.6 Software (Agilent) was used

for data acquisition and data analysis for assays. All plates were run at 37°C and samples were run in triplicate. The measurement of oxygen consumption and extracellular acidification method is as previously described in Choi et al. (2009). Oxygen consumption rates (OCR) and extracellular acidification rates (ECAR) were determined by sequential measurement cycles consisting of a 30 second mixing time followed by a 2 minute wait time and then a 3 minute measurement period. Reagents were added in Seahorse assay media in dilutions according to manufacturer's recommendation (2.0 µM Oligomycin, 1.0 µM FCCP, 0.5 µM Rotenone/antimycin A per well).

### ***Statistical Analyses***

Data were analyzed using GraphPad Prism Software (San Diego, CA) and R x64 version 3.5.1 for Mac OS10. Three- way Analysis of variance (ANOVA) were run in R to compare physiological changes associated with sex and diet across the 10 week diet and to compare sex and diet-induced changes observed in oxygen consumption rates at baseline and following the 10 minute forced swim test. Two-tailed unpaired t tests or two-way ANOVA's were used to analyze where significant main effects or interactions occurred ( $p \leq 0.05$ ) using GraphPad. Sidak's posthoc analysis was used when appropriate ( $p \leq 0.05$ ).

## Chapter Three

### Results

*A high fructose diet initiated in adolescence alters physiology in females, but not males*

Three-way ANOVA showed a main effect of sex ( $F_{(1,420)}=4071.63; p<0.001$ ), diet ( $F_{(1,420)}=14.26; p<0.001$ ), and week on the diet ( $F_{(9,420)}=1397.83; p<0.001$ ), as well as significant interactions between sex and diet ( $F_{(1,420)}=6.27; p=0.013$ ), sex and week ( $F_{(9,420)}=137.19; p<0.001$ ), and diet and week ( $F_{(9,420)}=2.42; p=0.011$ ) on weekly weight gain (**Figure 1.A**). All groups, regardless of sex and diet, gained weight over the course of the 10-week dietary window, consistent with expected animal growth. In females, individual 2-way ANOVAs displayed a main effect of week ( $F_{(9,180)}=429.3; p<0.0001$ ), diet ( $F_{(1,180)}=27.05; p<0.0001$ ), and a significant interaction between week and diet ( $F_{(9,180)}=3.615; p=0.0004$ ), such that fructose-fed females gained more weight per week than chow-fed controls beginning at week 7 (PND 73) and persisting through the remainder of the 10 week dietary period. In males, diet did not affect weight gain ( $F_{(1,240)}=0.3417; p=0.5594$ ). Caloric efficiency was calculated by dividing weight gained per week by the estimated amount of calories consumed per week. Caloric consumption was estimated from the total grams of food consumed per animal multiplied by the known caloric content of the respective diet (3.35kcal/gram of chow; 3.85 kcal/gram of HFD). Three-way ANOVA displayed a main effect of sex

( $F_{(1,378)}=315.03$ ;  $p<0.0001$ ), diet ( $F_{(1,378)}=16.96$ ;  $p<0.0001$ ), and week ( $F_{(8,378)}=487.35$ ;  $p<0.0001$ ). Three-way ANOVA also displayed a significant interaction between sex and diet ( $F_{(1,378)}=20.17$ ;  $p<0.0001$ ), sex and week ( $F_{(8,378)}=5.37$ ;  $p<0.0001$ ), and diet and week ( $F_{(8,378)}=3.08$ ;  $p=0.00226$ ) (**Figure 1.B**). Regardless of sex, caloric efficiency declined weekly. Diet did not alter caloric efficiency in males ( $p>0.05$ ). However, in females, two-way ANOVA demonstrated a main effect of week ( $F_{(8,162)}=136.4$ ;  $p<0.0001$ ) and diet ( $F_{(1,162)}=34.26$ ;  $p<0.0001$ ) with a significant interaction between week and diet ( $F_{(8,162)}=2.947$ ;  $p=0.0042$ ). Sidak's multiple comparisons test revealed that this interaction was driven by weeks 5 ( $p=0.0013$ ) and 6 ( $p=0.0492$ ), such that fructose-fed females in week 5 and 6 were gaining more weight per calorie consumed compared to their chow-fed counterparts. Unpaired t-tests displayed that in both males and females, circulating blood glucose was not affected by diet ( $p>0.05$ ) (**Table 1**).

*High fructose diet decreases anxiety-like behavior in males, but not in females in the open field*

In the open field test, two-way ANOVA displayed a main effect of sex ( $F_{(1,42)}=4.241$ ;  $p=0.0457$ ) in distance traveled (in centimeters) in the 10 minute open field test. When collapsed across diet, females traveled further than males (**Figure 2.C**). Neither sex ( $p>0.05$ ), nor diet ( $p>0.05$ ) affected the animal's time spent in the center of the arena as compared to the periphery (**Figure 2.B**).

However, frequency of crosses into the center was affected by diet in males. An unpaired t-test displayed a main effect of diet in males ( $p=0.0200$ ), such that fructose-fed males were crossing into the center of the arena more frequently than chow-fed controls. In females, number of crosses into the center was not impacted by diet ( $p>0.05$ ) (**Figure 2.A**).

*In both sexes, social behavior was not impacted by diet*

In the social interaction test, unpaired t-tests displayed that diet did not impact the latency of the experimental rats to approach the stimulus rats (**Figure 3.A**), the number of approaches of the experimental rats on the stimulus rats (**Figure 3.B**), or the total time rats spent interacting in the arena in both males and females ( $p>0.05$ ) (**Figure 3.C**).

*High fructose diet modifies behavior following the introduction of an acute stressor, in the form of a forced swim test*

Following the introduction of an acute stressor in the form of a 10-minute forced swim test, two-way ANOVAs were conducted assessing the impact of sex and diet. Two-way ANOVA displayed a main effect of sex ( $F_{(1,19)}=37.94$ ;  $p<0.0001$ ), demonstrating that males spent more time actively struggling in the tank than females (**Figure 4.C**). With regard to latency to inactivity, two-way ANOVA indicated a main effect of sex ( $F_{(1,19)}=7.378$ ;  $p=0.0137$ ), but no effect of

diet, such that males took a longer amount of time to cease activity in the tank (**Figure 4.A**). In addition, two-way ANOVA of time spent inactive in the forced swim test revealed a main effect of sex ( $F_{(1,19)}=20.94$ ;  $p=0.0002$ ) and diet ( $F_{(1,19)}=15.44$ ;  $p=0.0009$ ) and a significant interaction between sex and diet ( $F_{(1,19)}=6.271$ ;  $p=0.0215$ ). Further analysis using unpaired t-test displayed a main effect of diet in females ( $F_{(4,4)}=14.53$ ;  $p=0.0127$ ), such that fructose-fed animals spent more time inactive than chow-fed controls (**Figure 4.B**)

*Corticosterone concentration in rats were affected by acute stress event in males and females, and this effect was modified by diet in males*

In males, corticosterone levels were impacted by the experience of an acute stressor. Two-way ANOVA of male corticosterone concentration displayed a main effect of diet ( $F_{(1,17)}=8.66$ ;  $p=0.0091$ ) and acute stress experience ( $F_{(1,17)}=97.13$ ;  $p<0.0001$ ). Sidaks' multiple comparisons test revealed that regardless of dietary history, experience of an acute stress resulted in significantly higher corticosterone concentration (Chow,  $p<0.0001$ ; Fructose,  $p=0.0007$ ) (**Figure 5.A**). Further unpaired t-test revealed no main effect of diet in baseline males; however, in groups undergoing the forced swim test there was a main effect of diet ( $F_{(5,5)}=1.574$ ;  $p=0.0134$ ) such that fructose-fed animals had lower corticosterone concentrations than their chow counterparts (**Figure 5.A**). In females, two-way ANOVA revealed a main effect of acute stress history ( $F_{(1,15)}=22.89$ ;  $p=0.0002$ ), such that females experiencing an acute stressor

displayed higher corticosterone concentration (**Figure 5.B**). Diet did not impact corticosterone concentrations in females ( $p>0.05$ ) (**Figure 5.B**).

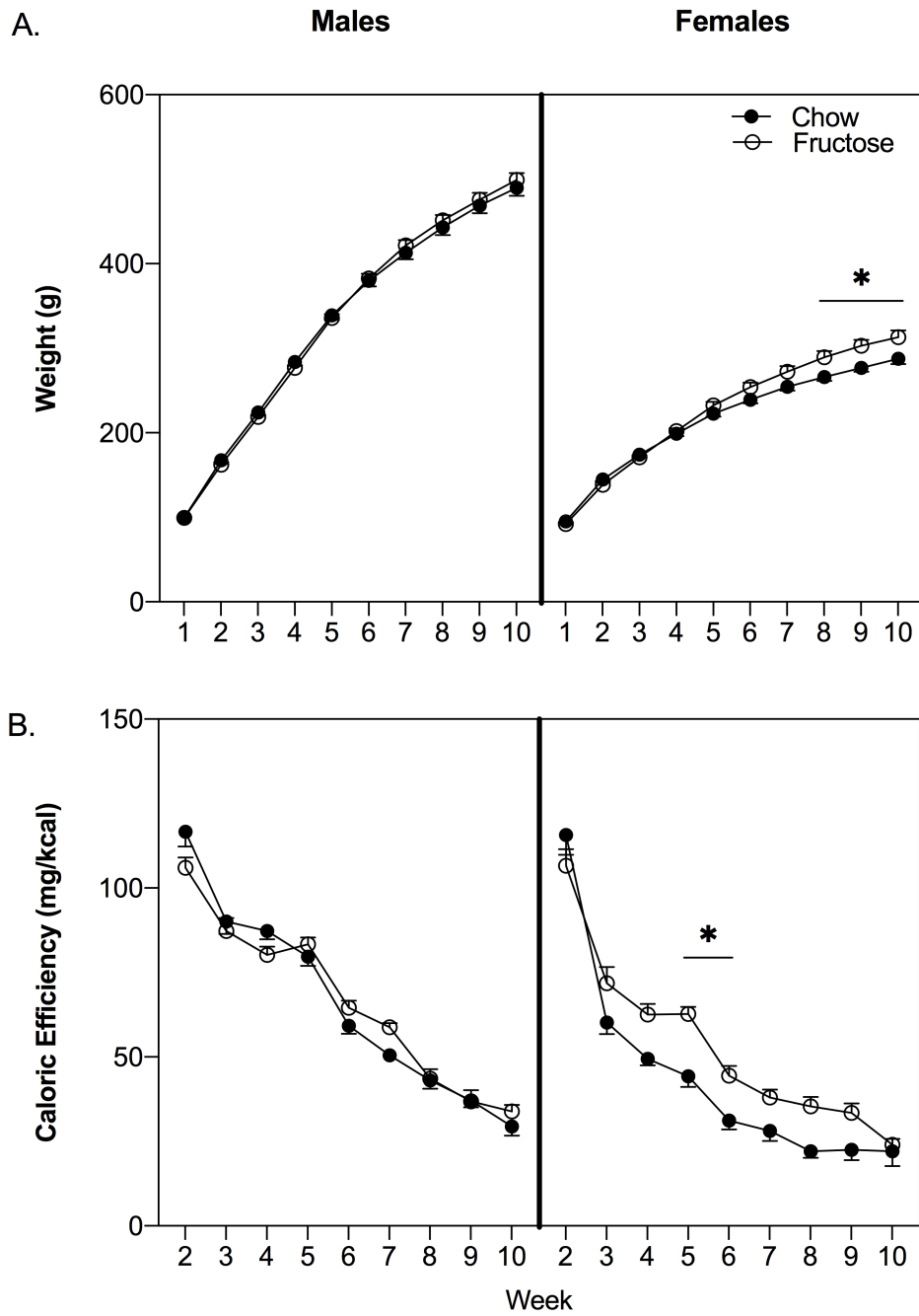
*High Fructose Diet impacts mitochondrial performance in both males and females*

Oxygen consumption rate (OCR) was modified by HFD at baseline and in the event of an acute stressor. Multi-factor ANOVA displayed a main effect of sex ( $F_{(1,312)}=8.00$ ;  $p=0.0049$ ), acute stress experience ( $F_{(1,312)}=24.36$ ,  $p<0.0001$ ), and measurement ( $F_{(11,312)}=60.29$ ;  $p<0.0001$ ). Significant interactions occurred between sex and diet ( $F_{(1,312)}=24.41$ ;  $p<0.0001$ ) and sex and acute stress experience ( $F_{(1,312)}=6.33$ ;  $p=0.0012$ ). Individual three-way ANOVAs were then conducted separating male data from female data. In males, there was a main effect of diet ( $F_{(1,180)}=14.28$ ;  $p=0.00021$ ), acute stress experience ( $F_{(1,180)}=28.28$ ;  $p<0.0001$ ) and measurement ( $F_{(11,180)}=38.66$ ;  $p<0.0001$ ). No significant interactions were present. Further two-way ANOVAs indicated that in both chow-fed and fructose-fed males there was a main effect of forced swim test ( $F_{(1,96)}=11.96$ ,  $p=0.0009$ ;  $F_{(1,84)}=16.58$ ,  $p=0.0001$ ) (**Figure 6.B**). Males undergoing the forced swim test demonstrated increased OCR compared to baseline controls, in both dietary groups. In addition, in both baseline groups and forced swim groups, there was a main effect of diet ( $F_{(1,84)}=8.967$ ,  $p=0.0036$ ;  $F_{(1,96)}=5.444$ ,  $p=0.0217$ ), such that fructose fed animals showed decreased OCR regardless of stress history (**Figures 6.B**).

In females, there was a main effect of diet ( $F_{(1,132)}=10.66$ ;  $p=0.00014$ ) and measurement ( $F_{(11,132)}=22.08$ ;  $p<0.0001$ ), but not of acute stress experience (**Figure 6.C**). Again, no significant interactions were evident. Two-way ANOVA revealed that the main effect of diet appeared in females undergoing the forced swim test ( $F_{(1,72)}=9.799$ ,  $p=0.0025$ ). Interestingly, fructose-fed animals undergoing the forced swim test demonstrated a higher OCR than chow-fed counterparts (**Figure 6.C**). Baseline females demonstrated no effect of diet ( $p>0.05$ )(**Figure 6.C**). Three-way ANOVAs conducted on individual indices of mitochondrial quality, including spare respiratory capacity and maximal respiration rate, displayed no main effect of sex, diet, or acute stress experience ( $p>0.05$ ).



Figure 1



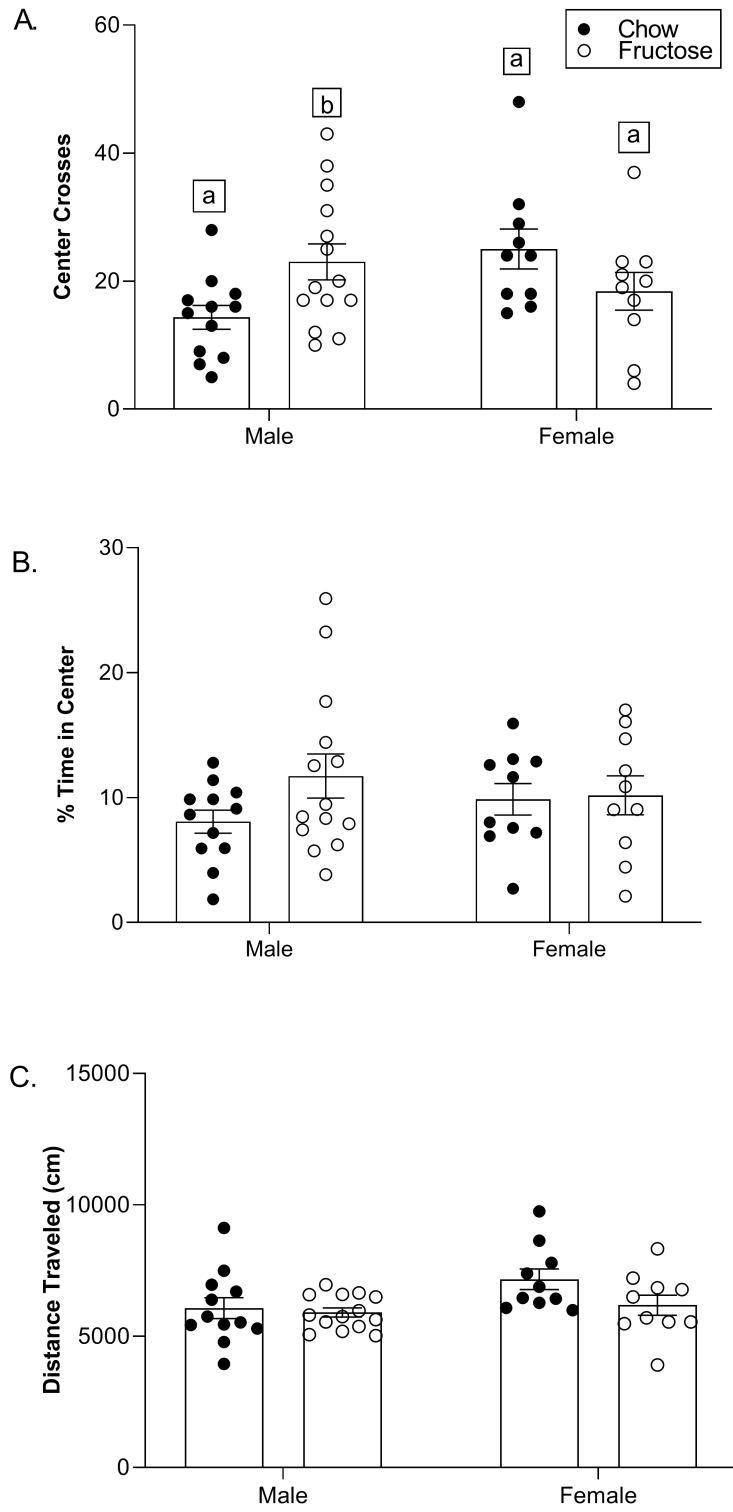
**Figure 1:** *An adolescent high fructose diet resulted in physiological differences in females, but not in males. A) Fructose-fed female rats gained more weight than their chow-fed counterparts beginning in week 8 of the dietary paradigm/ This effect was maintained throughout the 10 week consumption period. Symbols represent mean  $\pm$  SEM. \* $p < 0.05$ . B) The fructose diet altered caloric efficiency in female rats during weeks 5 and 6 on the diet. Alterations were not present in males. The caloric efficiency of both male and female rats decreased over time. Symbols represent mean  $\pm$  SEM. \* $p < 0.05$ .*

**Table 1**

Week on Diet	Males				
	Chow		Fructose		P Value
	Mean (mg/dl)	SEM	Mean (mg/dl)	SEM	
5	103.3	6.083	104.1	3.844	0.9074
10	76.67	3.278	83.21	2.219	0.1031
12	78.67	2.385	86.07	2.763	0.0577
Week on Diet	Females				
	Chow		Fructose		P Value
	Mean (mg/dl)	SEM	Mean (mg/dl)	SEM	
5	90.7	4.897	100.8	4.363	0.141
10	77.4	2.291	82.1	2.178	0.1544
12	82.2	2.682	84.9	1.76	0.4111

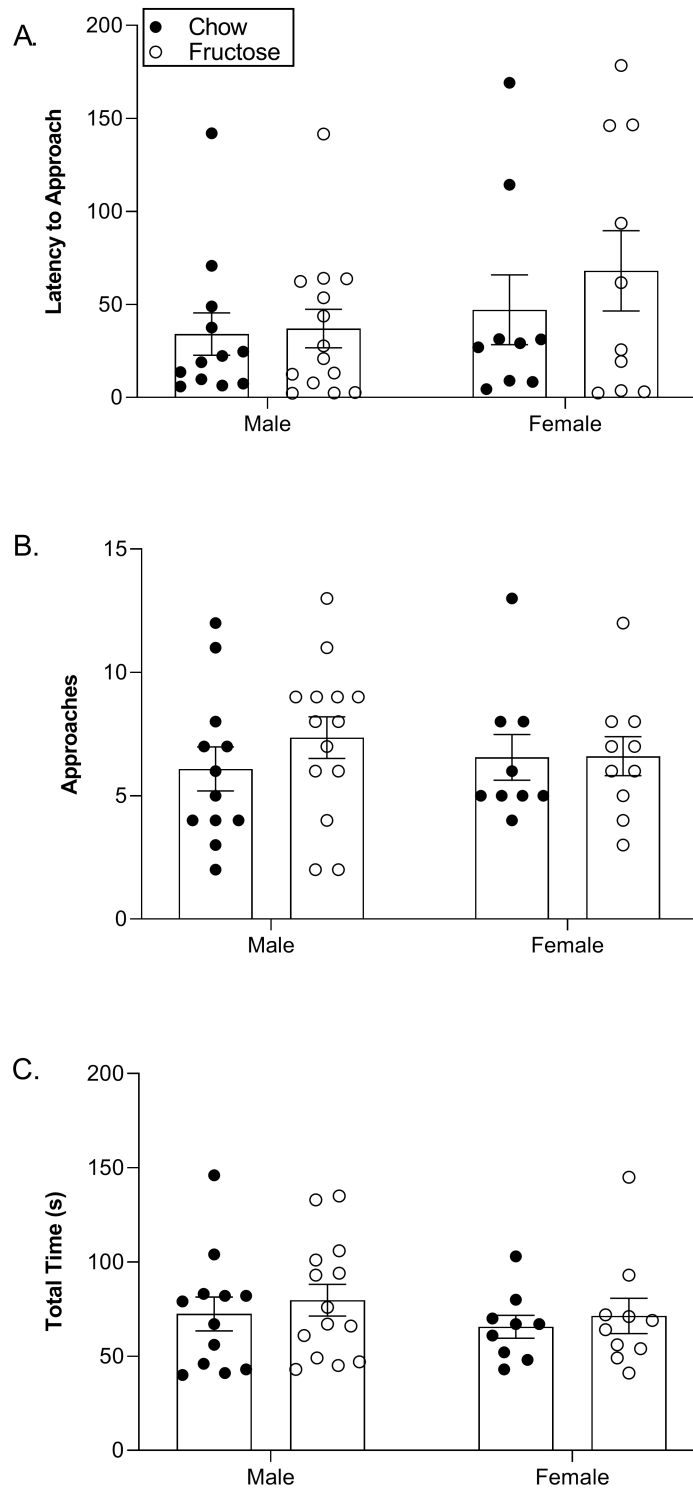
**Table 1:** *Consumption of a high fructose diet did not alter circulating blood glucose levels in male or female rats. Symbols represent mean  $\pm$  SEM.*

Figure 2



**Figure 2:** *Fructose consumption induced increases in exploratory behavior in male rats, but not in female rats.* A) Fructose-fed male rats demonstrated a high number of crosses into the center of the open field arena compared to chow-fed males. B) Amount of time spent in center of arena relative to the periphery was not altered by diet in males or females. C) Distance traveled in the open field was not modified by diet in either males or females. Symbols represent mean  $\pm$  SEM. Letters indicate effects with  $p < 0.05$ .

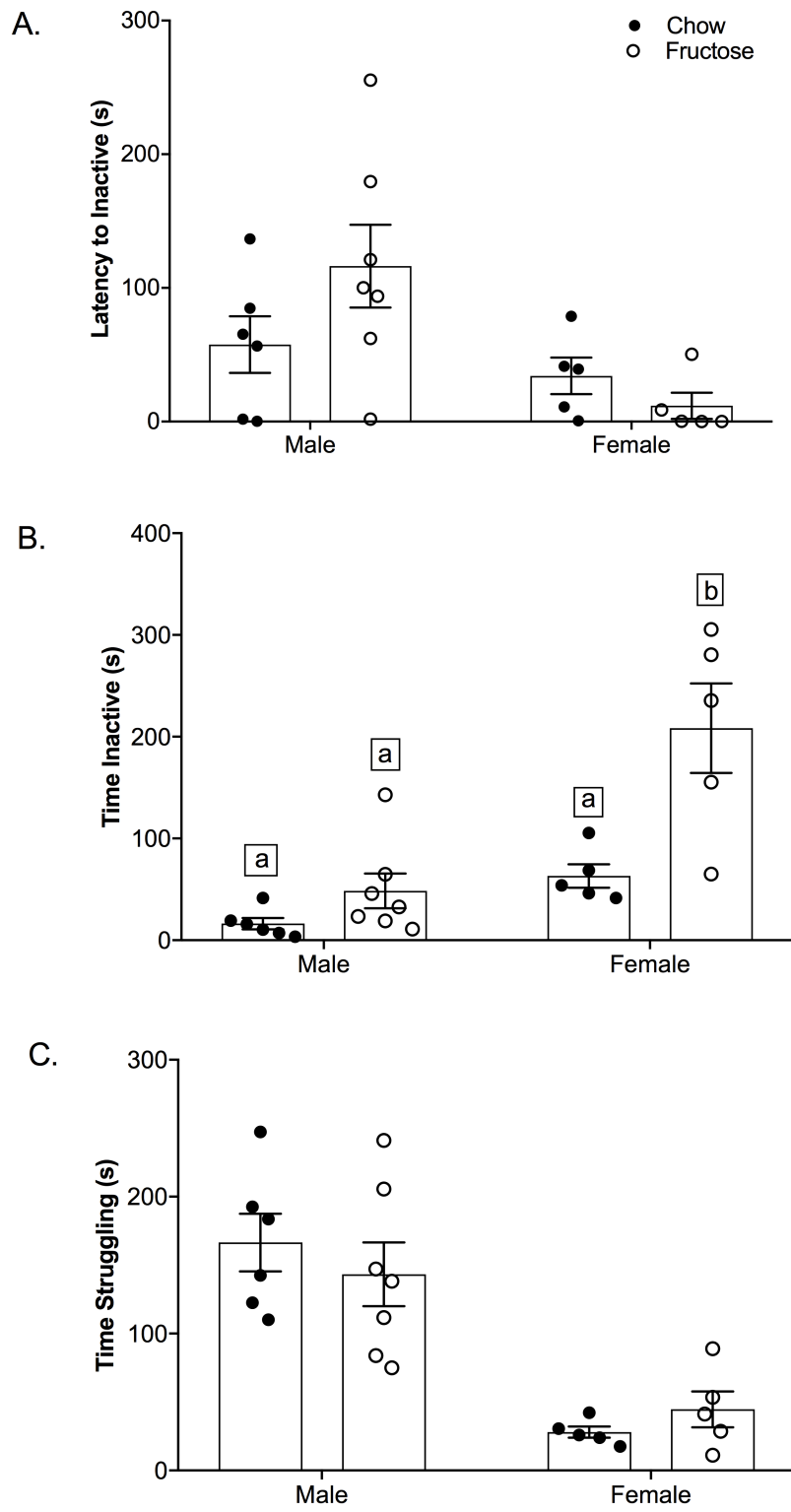
Figure 3



**Figure 3:** *In both sexes, social behavior was not affected by consumption of a high fructose diet. Symbols represent mean  $\pm$  SEM.*

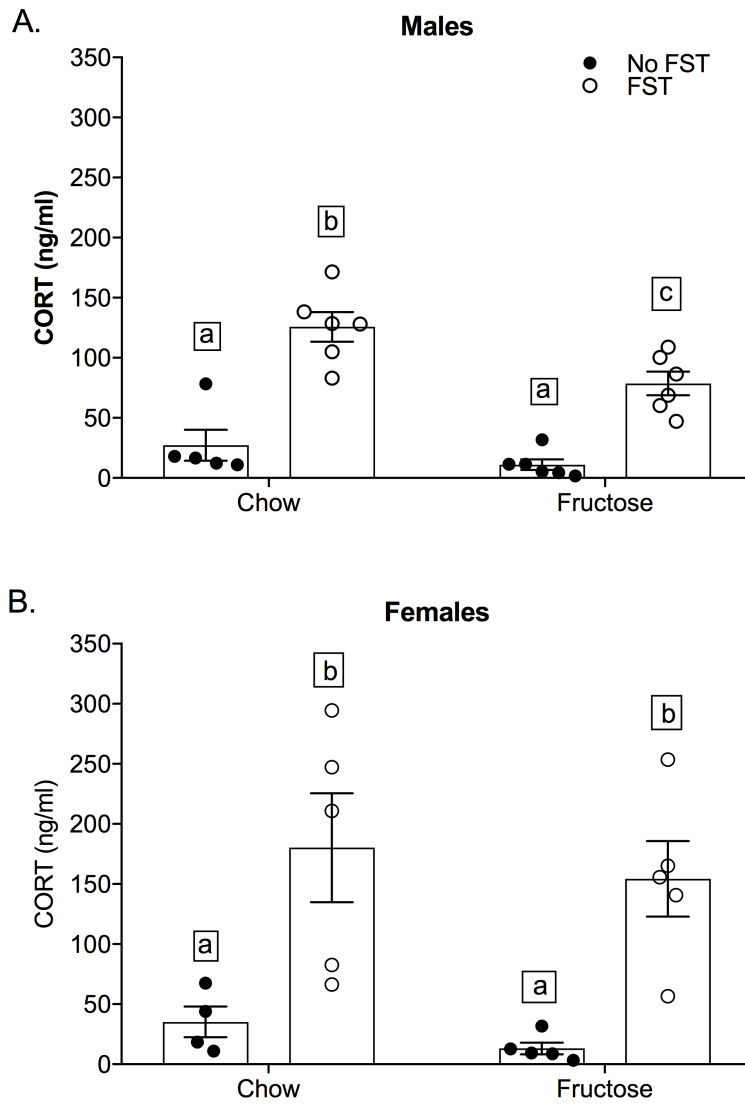


Figure 4



**Figure 4:** Consumption of a HFD resulted in increases in depressive-like behavior in the forced swim test in females. A) Latency to inactivity was not affected by diet in males or females in the forced swim test. B) Females fed a HFD spent more time inactive in the forced swim test than their chow-fed counterparts. Total time spent inactive in the tank was not modified by diet in males. C) Total time spent struggling was not modified by diet in males or females. Symbols represent mean  $\pm$  SEM. Letters indicate effects with  $p < 0.05$ .

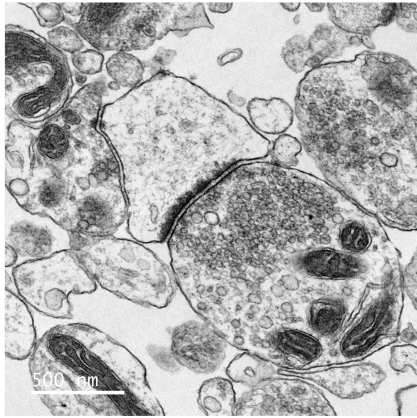
**Figure 5**



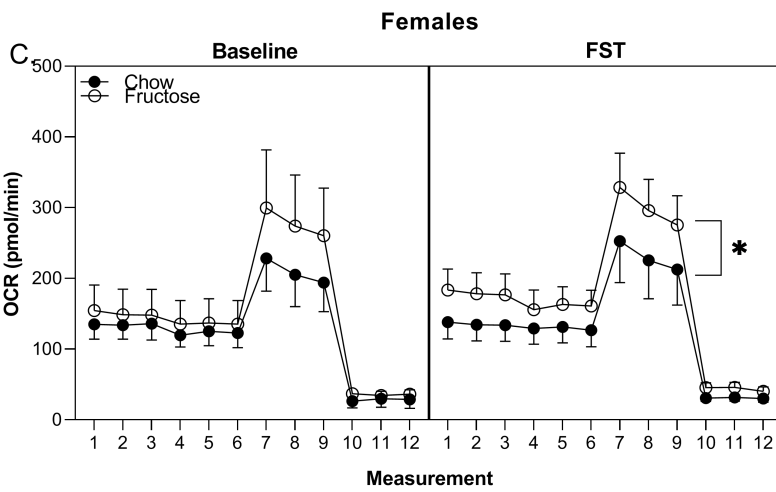
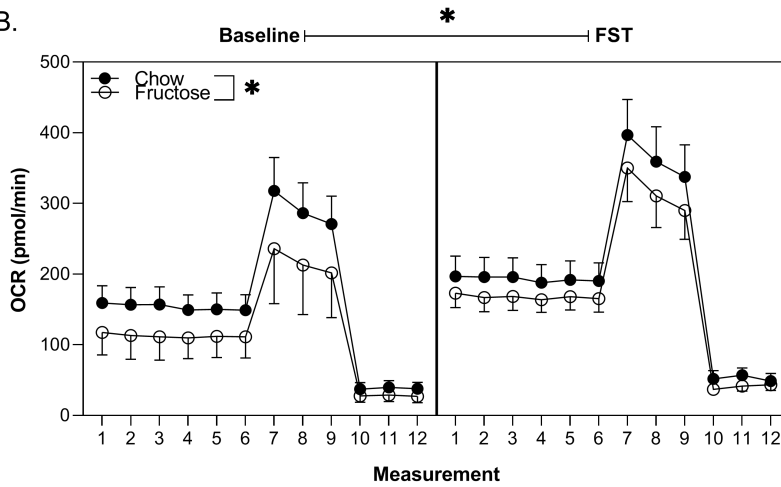
**Figure 5:** *Introduction of an acute stressor in the form of a 10 minute forced swim test increased blood corticosterone concentrations in both sexes, and this effect was modified by diet in males. A) In males, an acute stressor significantly increased corticosterone concentrations. In groups undergoing the forced swim test, corticosterone concentrations were decreased in those fed a HFD. B) In females, corticosterone concentrations were only modified by acute stress experience. Symbols represent mean  $\pm$  SEM. Letters indicate effects with  $p < 0.05$ .*

**Figure 6**

A.



B.



**Figure 6:** *In both sexes, mitochondrial respiration was modified by diet, but only by acute stress introduction in males.* A) Transmission electron microscopy image of a synaptosomal preparation collected in the lab. B) In males, acute stress experience significantly increased OCR in comparison to baseline groups. Regardless of stress experience, fructose-fed males demonstrated significantly lower oxygen consumption compared to chow-fed males. C) Acute stress experience did not affect mitochondrial respiration in females. In females undergoing the FST, fructose-fed females had significantly increased respiration rates compared to chow-fed females. Symbols represent mean  $\pm$  SEM. \* $p < 0.05$ .

## Chapter Four

### Discussion

Recently, more emphasis has been placed on investigating the deleterious effects of metabolic stress on behavioral and neurological outcomes. Excessive fructose consumption has been extensively characterized as a risk factor for peripheral epidemiology (Dupas et al., 2017; Sanchez-Lozada et al., 2007; Montonen et al., 2007). Adolescents are extremely vulnerable to overconsumption of fructose-laden foods, conferring a window of susceptibility during important developmental periods (Wang et al., 2008; Welsh et al., 2011). The findings in this study demonstrate that disproportionate consumption of fructose initiated in adolescence alters physiology, behavior, and synaptic mitochondrial respiration in a sex-specific manner.

#### ***Fructose consumption modifies physiological parameters***

Consumption of a HFD altered the physiological parameters assessed in this study in female rats, but not in male rats. Fructose-fed females gained more weight than chow-fed females beginning at 8 weeks on the diet. This is consistent with previous findings in our lab (Hyer et al., 2019, in review). Fructose is more lipogenic than glucose, leading to elevations in triglycerides and increased adiposity, possibly accounting for weight differences seen in female groups (Hallfrisch, 1990). Additionally, caloric efficiency was modified by diet in

weeks 5 and 6 of the paradigm in female rats, indicating that alterations in energy utilization are a possible source of the observed increases in body mass beyond that of the control-fed female rats. Literature reflects the ability of fructose to induce alterations in energy utilization when compared to glucose. In one clinical study males and post-menopausal females ingested either a high glucose diet or a HFD over the course of 10 weeks. Despite comparable increases in body weight, resting energy expenditure significantly decreased in subjects consuming fructose. This could result in weight gain if energy intake is not downwardly adjusted (Cox et al., 2012). Another study of short-term fructose ingestion in females reported that postprandial energy expenditure and thermogenesis was significantly increased in females consuming a bolus of fructose, compared to glucose (Schwarz et al., 1992). However, this study was conducted following a single meal of fructose, and did not cover the impact of a long-term dietary modification. Taken together, these studies elucidate that fructose metabolism initiates metabolic changes different from that of traditional glucose metabolism, and varying outcomes widely depend on experimental design.

Although weight, caloric efficiency, and blood glucose in males were not affected by diet, other studies in our lab have shown elevated circulating glucose and elevated circulating uric acid in males following a 10 week HFD, independent of weight gain (Hyer et al., 2019, in review). Traditionally, weight gain or alterations in caloric efficiency are the most superficial indication that metabolic alterations have occurred. However, the absence of modifications in body weight does not mean that maladaptive qualitative changes have not occurred with the



introduction of a HFD. Many studies have been conducted where ingestion of a HFD induced pathophysiological states indicative of metabolic syndrome independent of obesity (Tran et al., 2009; Hyer et al., 2019, in review; Harrell et al., 2015). Previously in our group, Harrell and colleagues notably demonstrated that while obesity did not occur in fructose-fed rats, increased weight of perirenal fat pads in fructose groups indicated a change in body composition based on diet (Harrell et al., 2015). Unfortunately, this metric was not assessed in the present study. During collections it was difficult to markedly distinguish perirenal fat pads from surrounding visceral fat and fat pads were not collected. If additional cohorts were generated, fat pad collection would be a priority during collections in order to confirm body composition changes.

### ***Affective-like behavior modifications following a HFD***

Chronic exposure to fructose resulted in anxiety-like behavioral modifications. In male rats, fructose-fed rats demonstrated increased central tendency in the open field, a validated measurement of anxiety-like behavior (Prut & Belzung, 2003). However, diet did not modify distance traveled or the percentage of time spent in the center relative to the periphery within the open field arena. This suggests that fructose consumption heightened exploratory behavior, thus representing a decrease in anxiety-like behavior in a novel environment. Indeed, this result is counterintuitive and contrary to previous findings. Harrell et al., previously demonstrated increases in anxiety-like behavior in the open field and alterations in the hypothalamic gene transcriptome in

fructose-fed male rats (Harrell et al., 2015). Another study by O'Flaherty and colleagues demonstrated no effect of fructose consumption on affective-like behavior, including within the open field, in male Sprague-Dawley rats (O'Flaherty et al., 2019). This is perhaps due to inherent genetic differences between strains. In addition, seemingly innocuous differences in environment could be responsible for differential behavioral outcomes. For example, differences in environmental enrichment have been shown to mitigate the effect of chronic stress on fear-conditioned behavior (Mitra & Sapolsky; 2009). Also, differences in bedding material and frequency of cage changes affected body weight and inflammatory markers in rodents (Yildirim et al., 2017). In one study, identical mouse strains demonstrated differing outcomes on 6 behavioral tests in 3 different labs despite extremely thorough attempts at standardizing protocols and controlling environmental factors (Simpson & Kelly, 2011). While the open field test in the present study reported opposite findings, it serves to recapitulate the malleability of the behavioral response in the face of a chronic metabolic disturbance. Interestingly, in this study fructose-fed female rats demonstrated no modifications in the open field test when compared to chow-fed females. Furthermore, neither males nor females demonstrated alterations in social behavior within the social interaction test.

In the forced swim test (FST), a validated measurement of depressive-like behavior (Porsolt et al., 1978), fructose-fed females exhibited increased floating time, indicative of a depressive-like phenotype. Importantly, increased floating time potentially demonstrates blunted ability to respond to a period of increased

energetic demand. The FST acted as an acute stressor, and in order to overcome this stress mobilized substrates are utilized to return to homeostasis. While the increase in floating time traditionally indicates an increase in depressive-like behavior, it may also dictate a disruption in metabolism, such that fructose-fed females are unable to efficiently perform the proper compensatory mechanisms to return to energetic homeostasis. Notably, fructose-fed males did not demonstrate increased floating time in the FST, as demonstrated in previous studies within our group (Harrell et al., 2015). However, there was a trend towards increased floating in fructose-fed males, but given that group sizes were at most 7 animals, groups may not have been large enough to detect differences at the 0.05 level. As it happens, power analysis of the FST revealed that sample sizes for each group would need to be 30 animals to reliably detect main effects. While this sample size is outside of what was possible for the present study, it does confirm that fructose-fed males were tracking in the direction of the previously reported data.

### ***Diet and acute stress experience altered glucocorticoid output***

The 10-week dietary window was initiated in adolescence, a particularly vulnerable time for development of the HPA axis (Gunnar & Donzella, 2001; Panagiotakopoulos & Neigh, 2014). As previously discussed, the HPA axis is the primary central regulator of energetic homeostasis in the body through the employment of hormonal cascades. The glucocorticoid, cortisol (corticosterone in rodents) has been of primary interest in the study of metabolic dysfunction due to

its ability to influence glucose homeostasis and energy mobilization, especially in response to times of biological and psychosocial stress. Thus, investigation into corticosterone concentrations provides an interesting bridge between behavior, HPA axis function, and metabolism. In males, modification of the HPA-axis was reflected via modifications to glucocorticoid output following the FST.

Measurement of corticosterone production following an acute stressor indicated that fructose-fed male rats produced significantly less corticosterone than chow-fed rats also experiencing the acute stressor, but both groups released significantly more corticosterone than baseline males, regardless of diet.

Interestingly, in this study fructose-fed female rats demonstrated increased depressive-like behavior when compared to chow-fed rats in inactivity in the FST. However, corticosterone response to an acute stressor was not modified by diet in females as it was in the male group. In the literature, depressive-like behavior is positively associated with increases in corticosterone concentrations (Ali et al., 2015; Johnson et al., 2006). The disconnect between the increase in depressive-like behavior and absence of modification in corticosterone concentrations in fructose-fed females indicates that a metabolic consequence of the HFD may be responsible for counteracting HPA axis dysfunction, which may be explained through the investigation of mitochondrial function. Unsurprisingly, both male and female rats displayed increased corticosterone concentrations following the FST when compared to baseline rats.

Excessive fructose consumption inflicts chronic energetic stress on the HPA-axis, resulting in the HPA-axis being unable to efficiently perform its

physiological role, and being less responsive to environmental stimuli. Works in the arena of chronic stress research support this concept. In the present study, male rats did not demonstrate increases in depressive-like behavior in the FST. In contrast, fructose-fed females displayed increased depressive-like behavior in the FST. However, previous work by our group has shown increases in depressive-like behavior in male rats following a HFD in the form of increased time inactive in the FST (Harrell et al., 2015). This provides an interesting context to the glucocorticoid response of the rats. Work by Peeters et al. (2003) investigated cortisol responses to negative and positive daily events in healthy and depressed patients, with an emphasis on gender differences. Depressed men showed blunted cortisol concentrations in response to a negative event, compared to healthy men. Additionally, depressed women demonstrated a larger cortisol response than depressed men to negative events (Peeters et al., 2003). Previous studies have reported blunted cortisol response to an acute stressor in healthy males with a history of chronic stress (Matthews et al., 2001). These clinical findings demonstrated that cortisol response to an acute stressor in women was unaffected by a history of chronic stress, suggesting that women are less responsive to environmental factors, such as a dietary disturbance, than men (Matthews et al., 2001). This possibly accounts for the fact that despite an increase in depressive-like behavior in the fructose-fed female rats, corticosterone concentrations were unaffected by diet. Alternatively, fructose consumption may have initiated divergent changes in metabolism in males and

females, necessitating different glucocorticoid responses in the face of an acute stressor.

### ***Acute stress experience alters oxygen consumption rates***

In this study, I chose to investigate metabolic modifications through the quantification of mitochondrial respiration in the brain. Acute stress experience impacted mitochondrial respiration in males, but not in females. Males undergoing the FST demonstrated increased oxygen consumption rate (OCR) regardless of diet when compared to baseline controls. While the FST represents an acute stressor, it also constitutes a period of increased energetic demand in order to overcome this stress. Due to this increased energetic demand it is perhaps unsurprising that mitochondrial respiration increased in response. Globally, mitochondria are particularly sensitive to challenges to homeostasis and adjust their bioenergetic output accordingly (Manoli et al., 2007). In response to stress, a cascade of hormones (such as corticosterone) is initiated in the CNS to mobilize substrates to meet the energetic demands of the 'fight or flight response'. These substrates are then available for oxidation by the mitochondria. Short-term exposure to elevated levels of glucocorticoids, as demonstrated by increased corticosterone concentrations following the FST, is associated with increased mitochondrial biogenesis and other enzymatic responses of respiratory chain complexes. In one study of skeletal muscle in male rats, Weber et al. demonstrated that short-term exposure of skeletal muscle cells to a synthetic glucocorticoid resulted in transcriptional stimulation of mtDNA, resulting in the

upregulation of mitochondrial biogenesis (Weber et al., 2002). Another study involving 24 weeks of chronic exercise and mice, revealed that following 24 weeks of chronic exercise, cytochrome oxidase, the enzyme that catalyzes mitochondrial oxygen uptake, showed increased activity in the brain of both male and female mice (Navarro et al., 2004). Taken together, these studies support the increased OCR in males undergoing the FST, as it was an acute period of energetic demand and increased substrate availability. In contrast, females undergoing the FST did not demonstrate significant modifications of OCR as a result of the FST. In a study investigating sex differences in mitochondrial biogenesis via mitochondrial protein synthesis in response to Sprint interval training, Scalzo et al. found that mitochondrial biogenesis was higher in males than females following 9 sprint interval training sessions over the course of 4 weeks (Scalzo et al., 2014). In conjunction with our studies, perhaps the 20-minute recovery period following the 10-minute FST was insufficient time in females to reflect changes in OCR as compared to males.

### ***Dietary modification of mitochondrial respiration***

Fructose-fed males demonstrated a decreased OCR in comparison to chow-fed counterparts, regardless of acute stress experience. This perhaps ties into the blunted response demonstrated in the corticosterone results, indicating that excessive consumption of fructose has hindered mitochondrial bioenergetic efficiency, possibly through reduced mitochondrial content or impaired mitochondrial function. This decrease in overall mitochondrial respiration may be

due to a number of factors. Metabolic syndrome and type 2 diabetes have been linked to specific mitochondrial tRNA mutations and other mtDNA abnormalities (Wilson et al., 2004; Patti et al., 2003; Nishio et al., 2004). Even excessive fructose consumption for as little as two weeks has been shown to increase inflammation markers, oxidative stress, and decreased mitochondrial oxygen consumption in the hippocampus of adult rats (Cigliano et al., 2018). Other studies have also dictated the negative impact of fructose consumption on mitochondrial function and increased oxidative stress (Mastrocola et al., 2016). This decrease could also be attributed to a variety of unique properties of the mitochondria. Dependent on their environment, mitochondria undergo dynamic changes to their morphology in order to regulate bioenergetics and energy efficiency. Under instances of acute stress, mitochondrial fusion occurs, resulting in interconnected and enlarged structures, which promote survival and energy expenditure. In contrast, mitochondrial fission results in fragmented and solitary mitochondria, signaling the cell for apoptosis. Mitochondrial fission is frequently observed in instances of long-term metabolic disturbances, such as in type 2 diabetes and metabolic syndrome (Liesa & Shirihai, 2013; Picard et al., 2014). However, at this time relatively little is known about the effect of diet on mitochondrial dynamics, especially in the context of brain tissue. Other studies have demonstrated that a HFD disrupts insulin signaling in the brain (Agrawal & Gomez-Pinilla, 2012). Insulin is a potent regulator of mitochondrial biogenesis and fructose's influence on this cascade could provide an explanation to decreased mitochondrial biogenesis as a result of excessive fructose



consumption. In addition, Agrawal et al. demonstrated that excessive dietary fructose reduced hippocampal mitochondrial OCR and reduced levels of proteins related to cellular energy metabolism (Agrawal et al., 2015). In conjunction with one another, these studies provide a number of possibilities to account for the decrease of OCR in fructose-fed males whether altered biogenesis, mitochondrial structure, or mitochondrial efficiency is at play.

With regard to diet, OCR was significantly impacted by fructose consumption in females. Contrary to findings in males, the HFD resulted in an increase in OCR in females undergoing the FST when compared to chow-fed females undergoing the FST. However, there was no effect of diet apparent in baseline females. In context of the brain, estrogen has been widely reported to provide a neuroprotective effect in the face of homeostatic challenges (Arevalo et al., 2015), including in the context of metabolic disorders (Carswell et al., 2000; Toung et al., 2000). Furthermore, several studies report that the neuroprotective effects of estrogen may act through specific mitochondrial mechanisms, such as hindering excessive ROS production, regulation of mitochondrial  $Ca^{2+}$  loading, and the preservation of mitochondrial membrane integrity during times of stress (Wang et al., 2001; Nilsen et al., 2003; Wang et al., 2006). Studies conducted in both male and female Wistar rats have reported that synaptic mitochondria of female rats produce less ROS than males, resulting in less oxidative damage to mitochondria (Sastre et al., 2004). Female resilience to mitochondrial damage may be responsible for the lack of effect seen in baseline females. In addition, the increase in OCR of fructose-fed females undergoing the FST relative to

chow-fed females provides interesting context to the lack of effect of diet on corticosterone concentrations seen in fructose-fed females undergoing the FST. Mitochondria contain powerful antioxidant machineries that assist in the body's defense against ROS. Additionally, many studies have documented the effect of known cytoplasmic antioxidants, such as resveratrol, on serum corticosterone concentrations and depressive-like behavior (Johnson et al., 2006; Liu et al., 2014). Increases in depressive-like behavior are associated with increases in serum corticosterone levels, such that corticosterone administration is often used to induce depressive-like behavior in rodents (Johnson et al., 2006) Given the upregulation of mitochondrial respiration in the fructose-fed females in the FST, it is plausible that this resulted in higher efficacy of mitochondrial antioxidant mechanisms, leading to the lack of dietary effect on corticosterone concentrations observed in these rats. Additional research is needed to confirm this hypothesis. It would be beneficial to investigate further markers of oxidative stress and antioxidant status, such as serum total antioxidant status and lipid peroxidation markers.

### ***Future Aims and Conclusions***

Further investigation is necessary to fully understand the implications of the data presented in this study. In order to confirm that physiological modifications have occurred in male and female subjects, remaining serum samples should be tested for elevated triglyceride levels. Chronic kidney disease is increasingly implicated in the manifestation of metabolic syndrome and type 2

diabetes. Therefore, kidneys collected from this cohort should be investigated for morphological changes under light microscopy such as vacuolar degeneration in the epithelial cells of proximal tubules (Bratoeva et al., 2017).

In regard to glucocorticoid investigations, fecal samples from the distal colon were collected from all animals in this study. Fecal corticosterone levels are not as readily susceptible to acute stressors as serum corticosterone levels (Thanos et al., 2009). Therefore, a fecal corticosterone ELISA could be used to confirm dietary modifications of glucocorticoid metabolites, and provide a more robust sample size when the FST is excluded as a variable.

Importantly, future studies are needed to explore the interaction between dietary fructose and synaptic mitochondria. The present study reveals that excessive fructose consumption decreases synaptic mitochondrial function in males and increases respiration in fructose-fed females in the FST. However, the specific mechanisms of these actions are yet undefined. Due to the lack of significance seen in individual mitochondrial indices, it is probable that overall quantity of viable mitochondria is somehow modified following the HFD. Staining and imaging of mitochondria with fluorescent probes would be useful in looking at mitochondrial morphology and quantity to compare whether fructose consumption promotes mitochondrial fission or led to mitochondrial density changes in synapses. Other studies report sex differences in synaptosomal functional studies. Mitrovic et al. reported that glutamate and aspartate transport in synaptosomes was significantly impacted by cycle stage in females (Mitrovic et al., 1999). Also, Oztas et al., demonstrated significantly decreased synaptosomal

Na(+)/K(+)ATPase activity and increased BBB permeability following water intoxication in females (Oztas et al., 2000). These studies support the idea that mitochondrial respiration is significantly impacted by sex, and the data presented here dictate the necessity of maintaining sex as a biological variable in future studies. As previously discussed, it would be useful to quantify antioxidant levels, such as glutathione reductase and glutathione peroxidase, and markers of oxidative stress, such as malondialdehyde (MDA) and reduced glutathione, in brain tissue. Additionally, resveratrol administration has been shown to provide a neuroprotective effect against oxidative stress (Sinha et al., 2002) and to reverse depressive-like behavior following chronic unpredictable stress (Liu et al., 2014). Therefore, I believe that mitochondrial respiration assessment following administration of a known antioxidant, such as resveratrol, would provide mechanistic insight into the dietary modifications shown in this study.

Given that hypothalamic gene expression in males was previously demonstrated in conjunction with altered affective-like behavior and hormonal outcomes, it would be interesting to see if similar alterations occur in the female transcriptome. More specifically, a mitochondria-neuron focused microarray could be performed in future cohorts to provide greater insight into the differential pathways underlying dietary modifications with specific connections to mitochondrial dysfunction (Su et al., 2011). This assay would be particularly prudent in the investigation behind the increase in mitochondrial respiration demonstrated in fructose-fed females in the FST, considering that the available literature does not provide much evidence to explain this observation.

In conclusion, we expand on previous studies conducted on male rats to include female rats in the study of how a HFD initiated in adolescence impacts affective-like behavior and physiology. The data presented corroborate the potential of excessive consumption of fructose to induce physiological changes, alterations in anxiety-like and depressive-like behaviors, and alters hormonal outcomes. I also demonstrate, for the first time, that a high fructose diet induces sex-specific alterations in mitochondrial metabolism. Mitochondrial respiration quantification presents an interesting cellular mechanism to connect the energetic state of an organism to long-term health outcomes on many levels.

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## Vita

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