Varied levels of fructose consumption induce physiological, cognitive, and mitochondrial alterations in aged female rats

Emilie L. Bjerring

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Varied levels of fructose consumption induce physiological, cognitive, and mitochondrial alterations in aged 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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B.S. Psychology, William & Mary, 2020

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Abstract

VARIED LEVELS OF FRUCTOSE CONSUMPTION INDUCE PHYSIOLOGICAL, COGNITIVE, AND MITOCHONDRIAL ALTERATIONS IN AGED FEMALE RATS

Emilie Louise Bjerring

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, 2022

Gretchen N. Neigh, Ph.D., Associate Professor, Department of Anatomy and Neurobiology

Since the 1970s, fructose consumption has dramatically increased within the United States, as well as the world. While adolescents tend to be the largest consumer of fructose, mostly seen in the form of sugary beverages, the consequences of a high fructose diet started in adulthood can also have severe implications on physiological parameters as well as cognition. Several studies have linked fructose consumption to metabolic syndrome, a clustering of symptoms related to overall health, with particular emphasis placed on obesity, type II diabetes, and the relationship with Alzheimer’s Disease. These findings largely stem from the outcomes of studies on cognition, both in humans and rats, assessing the extent to which fructose consumption alters cognitive flexibility. Aging alone is a factor in cognitive decline, yet the extent to which age interacts with diet is largely unknown. Additionally, more emphasis has been placed on uncovering the relationship between diet and mitochondrial respiration as a possible
explanation for sex-specific and age-related differences seen in relation to metabolic stress. Mitochondria are particularly vulnerable to metabolic disturbances, and as such, synaptosomal mitochondria in the hippocampus and prefrontal cortex were analyzed in this study. Surmountable evidence can conclude the effects of a fructose-rich diet on the cognition, behavior, and mitochondrial respiration of male rats, yet females are often neglected from studies. Males and females are equally susceptible to the deleterious effects of fructose, but the manifestation of these outcomes differ significantly between the sexes, according to research from our group. Several theories illuminate estrogen as a neuroprotective hormone that allows females to resist the deleterious effects on cognition, which can explain the negative implications of a high fructose diet being displayed in post-menopausal women only. However, females do seem to be more susceptible to physiological perturbations, and as such remains a point of interest. I therefore determined the extent to which a high fructose diet (55% fructose – 55FD) and a medium fructose diet (18% fructose – 18FD) differentially impacted the physiological parameters, cognition, and mitochondrial respiration of 12-month-old (aged) female rats. Additionally, I examined the potential of estrogen as a neuroprotective factor by administering a high fructose diet to ovariectomized and non-ovariectomized 6-month-old female rats. In the first experimental group, the 18FD group showed significantly higher body weights than their counterparts, while amount consumed and caloric efficiency was not significantly distinct. Additionally, all diet groups showed cognitive rigidity, and the 18FD group displayed increased levels of OCR in the hippocampus. In the second experimental group, there were no implications that
estrogen/estradiol played a significant role in protecting non-ovariectomized females from the deleterious effects of a high fructose diet.

In this thesis, I will outline current literature on fructose, how it is metabolized, and the associated outcomes of consuming fructose-rich diets. In addition, I describe the experimental set-up and the assessments performed in order to demonstrate the effects of fructose on physiological, cognitive, and mitochondrial function in female Wistar rats. I then describe the results and finally discuss the interpretation of these results as well as highlight potential future directions for this research. This thesis should aid in further illuminating the consequences of consuming fructose in adult females, the extent to which cognition and associated diseases are affected by mitochondrial dysfunction, and the role estrogen plays in sheltering this effect.
Chapter One

Introduction

Often nicknamed the “Western Diet,” the intake of fructose, in multiple forms, has been steadily increasing over the past two to three decades (Havel, 2005). This increase can largely be attributed to added sweeteners such as sucrose and high-fructose corn syrup, particularly seen in commercialized food products and sugary beverages (Marriott et al., 2009). As of 2013, over 74% of all foods contained some amount of added sugars (Bray & Popkin, 2013) accounting for approximately 16% of all caloric intake (Bray et al., 2004). In the present study, I review data related to the contributions of fructose to metabolic syndrome as well as neurobiological and behavioral consequences, which is likely driven by the way fructose is metabolized. Interestingly, several publications have revealed a neuroprotective effect of estrogen on cognitive decline, particularly in the context of homeostatic challenges and metabolic syndrome (Arevalo et al., 2014; Carswell et al., 2000). I hypothesized that consumption of fructose at varied levels beginning in adulthood would differentially impact metabolic function and cognitive behavior of 1-year-old (aged) female rats. Additionally, I hypothesized that these alterations would be paralleled by increases in mitochondrial respiration, given previous literature indicative of increased oxygen consumption rate (OCR) in females (Kloster et al., 2021).

Further, taking into account the effects of estrogen, I hypothesized that fructose consumption beginning in adulthood in ovariectomized (OVX) rats would result in deleterious effects on metabolism and increased cognitive rigidity when compared with
non-OVX counterparts. Moreover, I hypothesized that these effects would be highlighted by alterations in mitochondrial respiration. Collectively, an understanding of the impact of fructose, and to some extent estrogen, on metabolism, behavior, and neurology will build a framework within which solutions to fructose-altered consequences can be ascertained. In addition, these data will bring awareness to the long-term implications of consuming fructose and the differential impact of such a diet on those with higher levels of estrogen.

Fructose background and metabolism

From an evolutionary perspective, there is a natural reward that comes from eating sugar, even surpassing the addictiveness of cocaine (Ahmed et al., 2013). Increased consumption of foods high in sugar (such as ripened fruit and honey) would have increased the chances for survival during periods of food scarcity, as sugar helps to lay down fat, and when found in nature, generally indicates ample amounts of calories for energy (DiNicolantonio et al., 2017). Currently, inhabitants of the United States consume almost twice as much sugar as the French (Goldfein & Slavin, 2015), and more than one-third of adults and 17% of youth are obese in the US (Goldfein & Slavin, 2015). In the early 1970s, high fructose corn syrup was first introduced and is still the dominant caloric sweetener consumed in the American diet. It is easy to produce, made from corn, and is an inexpensive alternative to sugars. It was first developed as a liquid alternative to sucrose using a glucose isomerase to hydrolyze cornstarch into glucose and then further isomerize glucose into fructose (Parker et al., 2010). High fructose corn syrup consistently ranks among the top as an additive due to
its sweetness, solubility, acidity, and its relative cheapness in the US (Parker et al., 2010). The development of high fructose corn syrup has proven to be extremely beneficial to the manufacturing industry, yet rather concerning for the health of the general public consuming this product.

The ways in which glucose and fructose are metabolized by the body differ significantly. Generally, the body utilizes glucose as its primary source of energy, yet the overabundance of fructose can create severe alterations in energetic homeostasis. At the molecular level, fructose and glucose possess the same molecular formula (C6H12O6), but differ in one chemical group – namely the glucose possessing an aldehyde group on the carbon chain and fructose a ketone group. Additionally, fructose and glucose are differentially absorbed in the intestinal tract, with glucose being absorbed higher in the small intestine (Havel, 2005). Upon absorption by the intestinal epithelium, fructose is transported to the hepatic portal vein where it is preferentially metabolized by the liver. Once absorbed, fructose is rapidly phosphorylated by ATP to fructose-1-phosphate, catalyzed by fructose kinase. It then bypasses the rate-limiting step of glycolysis catalyzed by phosphofructokinase (Havel, 2005) and is more rapidly converted into glucose, glycogen, lactate, and lipids (Havel, 2005; Rutledge & Adeli, 2008).

There are additional consequences of consuming fructose, particularly relating to the endocrine system and the manifestation of metabolic perturbations as a result. Unlike glucose, accumulating evidence indicates that fructose intake is strongly associated with development of hepatic insulin resistance, even when total energy intake is matched by equal calories from glucose (Softic et al., 2020). This is due to the
fact that once fructose is absorbed, pancreatic beta cells are prevented from releasing insulin, which in turn means that insulin-regulated hormones leptin and ghrelin are unable to be modified (Maiztegui et al., 2010). In a seven-day study of hypercaloric feeding, hepatic glucose production was significantly increased and lowered whole body insulin sensitivity, when compared to the standard diet-fed group (Lê et al., 2009). Leptin is often referred to as the “satiety hormone” because it rises in response to insulin, while ghrelin, the “hunger hormone,” decreases (Teff et al., 2004). Leptin resistance due to fructose consumption has been linked to the development of non-alcoholic fatty liver disease (NAFLD), a disease increasingly associated with metabolic syndrome (Softic et al., 2020; Paschos & Paletas, 2009). This hormonal dysregulation has the potential to result in excessive caloric intake, which contributes to a multitude of metabolic disorders, most notably obesity and metabolic syndrome.

However, fructose-induced excessive consumption of energy is not the only proposed mechanism by which a diet high in fructose disturbs metabolic homeostasis. Several studies have indicated the presence of metabolic disturbances, such as fatty liver disease and metabolic syndrome, independent of excessive caloric intake (Gersch et al., 2007; Roncal-Jimenez et al., 2011). Stanhope et al. reported that consumption of 25% of daily energy requirement from fructose-sweetened beverages for ten weeks was enough to increase blood glucose, fasting insulin, and insulin excursion (Stanhope et al., 2009). Taken together, these data suggest that while fructose consumption can induce excessive caloric intake resulting in a variety of metabolic disturbances, differential metabolism of fructose alone may be enough to contribute to the deleterious consequences of a high fructose diet.
Metabolic Syndrome

Several studies have found that high fructose consumption has contributed significantly to an epidemic of metabolic syndrome (Havel, 2005) with rising incidence of obesity and Type II diabetes (Elliott et al., 2002). Metabolic syndrome has existed for at least 80 years and is defined widely as a clustering of several cardiovascular disease risk factors, including insulin resistance, glucose intolerance, prothrombosis, hypertension, central obesity, and dyslipidemia (Eckel et al., 2005). The epidemic correlates with pronounced changes in environment, behavior, and lifestyle and is considered one of the main threats to human health worldwide (Nakagawa et al., 2006). Thus, fructose is an important focus of study both because of the epidemiological evidence and as proof of principle to understand how peripheral metabolic changes drive shifts in localized neural metabolism which may underlie multiple neuropsychiatric disorders.

A recent study of metabolic syndrome among US adolescents revealed that impaired reading along with impaired memory and attention scores was a common effect of excessive fructose ingestion (Rubens et al., 2016; Privitera et al., 2011). In addition, elevated serum uric acid predicts the development of obesity and hypertension (Masuo et al., 2003), which raises the possibility that uric acid may have a pathogenic role in metabolic syndrome (Nakagawa et al., 2006). In a study on male Sprague-Dawley rats, some were assigned a standard control diet while others were placed on a 60% fructose diet. After four weeks, blood samples were obtained and half of the fructose rats were given allopurinol for an additional six weeks. Results indicated
that serum uric acid levels, systolic blood pressure, and fasting insulin levels were all elevated in the fructose-fed rats in addition to a relative increase in body weight. Rats that were given allopurinol showed an effective lowering of uric acid and therefore improvement in metabolic syndrome (Nakagawa et al., 2006). Recent work from our lab has been able to confirm these findings, using male and female Wistar rats to uncover the link between elevated serum uric acid and the induction of metabolic syndrome (Hyer et al., 2019). Additional studies from our lab have demonstrated that a diet high in fructose induces hyperglycemia (Harrell et al., 2015), increases depressive-like behaviors (Kloster et al., 2021;Harrell et al., 2015), alters cognitive performances, alters stress responsivity (Harrell et al., 2015), increases peripheral (Harrell et al., 2018) and central (Harrell et al., 2015;Harrell et al., 2018) inflammation causes liver damage (Kloster et al., 2021), and modifies synaptosomal mitochondrial function (Kloster et al., 2021). Recent studies in humans have shown that fructose consumption is associated with the development of hepatic and adipose tissue insulin resistance, and dyslipidemia due to its ability to induce hepatic de novo lipogenesis (Goldfein & Slavin, 2015).

**Effects of fructose on an aging population**

Adolescents continue to be the highest consumers of fructose, particularly through the consumption of sugary beverages, and a recent study has indicated that 1 in 5 adolescents develop pre-diabetes putting them at increased risk for metabolic disorders (Kuehn, 2020). While dietary habits established in childhood are likely to continue into adulthood, previous research from our group has established that a high-fructose diet (HFD) initiated in adolescence has distinct effects from consumption
in adulthood (Harrell et al., 2015). In adults, excessive ingestion of fructose is associated with an increased risk of diabetes and metabolic syndrome (Bazzano et al., 2008; Montonen et al., 2007). According to the World Health Organization, in 2016, the United States ranked as the most obese OECD country in the world, with 36.2% of the adult population considered obese. However, in addition to the somatic effects of diabetes and metabolic syndrome described, these conditions are highly comorbid with depression and cognitive dysfunction (Rubens et al., 2016), and type II diabetes is seen as a risk factor for Alzheimer’s disease (Moreira et al., 2013). These psychological disorders are more strongly associated with the adult population and are tightly linked with the excessive consumption of fructose.

Recently, our group has established diet-induced and age-specific changes in neurometabolism, specifically oxygen consumption rate in the synaptosomal mitochondria (Kloster et al., 2021; Hyer et al., 2022). In the Hyer et al. study, OCR was significantly altered among 3-month-old (young), 6-month-old (mid aged), and 12-month-old (aged) rats. Namely, among the females, OCR was highest in the mid aged group and lowest among the aged group in the hippocampus of both hemispheres (Hyer et al., 2022). Additionally, the Kloster et al. study revealed increased levels of OCR in the HFD group of females, yet decreased levels among the males (Kloster et al., 2021). In terms of behavior, Kloster et al. demonstrated a sex difference in that female rats did not show cognitive decline, which has been confirmed in other studies from our group (Kloster et al., 2021; Hyer et al., 2018). Taking these data into account, it is evident that age plays a factor in neurometabolism which can be altered through the induction of a fructose-filled diet. This resistance to cognitive decline in females could
be due to the presence of estrogen, since diet-related decline is only evident with menopause (Cournot et al., 2006).

**Behavioral and cognitive effects of fructose**

It is understood that excessive fructose intake results in perturbations of peripheral metabolism and contributes to pathophysiological outcomes such as metabolic syndrome. However, the neurological consequences of such a diet are less defined in the literature, limiting the development of therapeutic strategies. In a direct sense, fructose triggers systemic inflammation, induces neuroinflammation, brain oxidative stress, and other metabolic perturbations through inflammatory cytokines and/or plasma metabolites into the CNS (Spagnuolo et al., 2020). Fructose is absorbed in the small intestine by glucose transporter 5 (GLUT5) and then transported from enterocytes into systemic circulation by GLUT2 to be metabolized in the liver and kidney (Holdsworth & Dawson, 1964). A study on mice revealed that fructose not cleared by the liver and kidney can reach the brain through the Blood Brain Barrier (BBB), which contains GLUT5 receptors (115) allowing the brain to utilize fructose as a source of energy (Jang et al., 2018).

Recently, there has been increased focus on the ability of fructose to induce neuroinflammation, resulting in psychological stress. Four weeks of fructose feeding induced increased expression of the proinflammatory mediator genes Interleukin (IL)-1beta, IL-6, and Tumor Necrosis Factor (TNF)-alpha, together with Toll-like receptor 4 (TLR4), myeloid differentiation factor 88, and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB), in the hypothalami of rats (Hsu
Fructose feeding was shown to be associated with increased levels of histone deacetylases 3 (HDAC3) suggesting that it represents a crucial component in the network linking fructose to neuroinflammation in metabolic syndrome, through the activation of the TLR4/NF-κB pathway (Mielke et al., 2005). These pro-inflammatory effects have been found in other regions of the brain in rodent models.

As mentioned previously, glucose and fructose are both monosaccharides with the same number of calories and chemical formula, however they are metabolized differently. While glucose tends to circulate in the blood, fructose is extracted into the liver, where unregulated fructose metabolism can lead to lipogenesis (Luo et al., 2015). Interestingly, in humans, ingestion of glucose has been shown to reduce hypothalamic cerebral blood flow (CBF), a marker of neural activation, which is not seen upon ingestion of fructose (Page et al., 2013). Following the ingestion of a glucose bolus, functional Magnetic Resonance Imaging (fMRI) revealed a significant reduction in hypothalamic CBF compared to the increase seen upon ingestion of a fructose bolus (Zhao et al., 1999). Additionally, the study found that glucose consumption encouraged deactivation of the hypothalamus, thalamus, and striatum, whereas fructose ingestion only revealed deactivation of the hypothalamus and the thalamus (Page et al., 2013).

One of the more profound neurological implications of a diet high in fructose is the increasing amounts of evidence linking fructose consumption to an increased risk of developing Alzheimer’s Disease (AD). In 2005, a study on hamsters revealed that fructose was associated with a reduction in the tyrosine phosphorylation of the insulin receptor, as well as the serine/threonine protein kinase B (Akt) activity in the brain (Spagnuolo et al., 2020). In addition, the study demonstrated a significant increase in
the levels of the protein tyrosine phosphatase 1B, which contributes to neural insulin resistance by dephosphorylating components of the insulin cascade (Mielke et al., 2005). Insulin resistance has been closely associated with the progression of AD. In a more recent study, rats receiving 10% fructose in drinking water for 10 weeks developed a hypothalamic insulin signaling defect along with dysfunction of 3 inflammasome pathways in the hypothalami of the brains (Zhang et al., 2014).

In addition to and as a consequence of the cognitive implications of a HFD, there are several behavioral perturbations that can be noted. In a study done on male Sprague-Dawley rats, a 60% fructose diet was administered before testing learning and memory on the Water Maze Task (Zhao et al., 1999; Ross et al., 2009). Results indicated that while initial learning was not impaired in any group, the HFD rats saw significant deficits in retention, likely due to deficits in their hypothalamus (Ross et al., 2009). These findings are consistent with the literature regarding male rats, yet our lab has shown significant sex differences in relation to tasks that assess learning and memory. In Hyer et al., male rats on a HFD show significantly more cognitive rigidity on the Barnes Maze Assessment, whereas the females seem almost protected from this effect (Hyer et al., 2018). Additional work by Harrell et al. further demonstrates this effect (Harrell et al., 2015). Fructose inherently impacts a multitude of neural systems as well as affects the cognition of male rats.

**Metabolism and mitochondria**

Given that fructose affects both behavior and cognition in addition to the peripheral metabolic perturbations mentioned, it is important to consider the role of
fructose in mitochondrial respiration. Mitochondrial respiration is an important aspect of neural function and often reflects the implications of behavioral or cognitive alterations. All mammalian cells contain mitochondria in order to provide the energy, in the form of ATP, that is necessary for reactions to occur. Additionally, mitochondria are responsible for critical functions related to cell health such as calcium buffering, reactive oxygen species (ROS) production, and antioxidant mechanisms (Wang et al., 2001; Nilsen, 2008). Importantly, mitochondria are particularly sensitive to the metabolic state of an organism and as such, have been shown to be severely affected by a high fructose diet (Kloster et al., 2021; Harrell et al., 2015; Hyer et al., 2018), and may even be involved in the development of AD (Swerdlow, 2018). The overloading of mitochondria through the intake of excessive calories has been shown to increase fragmentation and increase production of ROS (Picard & Turnbull, 2013).

In the present study, particular focus was placed on the mitochondria of the synaptosomes in the hippocampus as well as the prefrontal cortex. Both of these brain regions play significant roles in assessing cognitive function – particularly, the hippocampus is associated with memory, while the prefrontal cortex assesses the cognitive flexibility of the organism (Voss et al., 2017; Heisler et al., 2015). It is the job of the mitochondria to provide enough ATP to effectively release neurotransmitters at the synapses, which is why OCR is a measurement indicative of the function of the synaptosomal mitochondria (Rangaraju et al., 2014). Normally, mitochondria produce ROS during ATP production, and in small amounts, provide the ROS necessary for cellular function, cellular differentiation, immunity, and intracellular signaling. The problem arises when a state of oxidative stress induces excessive ROS production
leading to impaired activity, decreased synaptic connectivity, and eventual apoptosis (Allen et al., 2018).

Previous studies have highlighted that environmental factors such as extreme stress translate to neural metabolic dysfunction through oxidative damage that can impair neuronal integrity, alter neuronal mitochondrial trafficking, and modulate mitochondrial function (Hunter et al., 2016) inducing a global change in neural metabolism (Turkson et al., 2019; Picard et al., 2018). Additionally, Hyer et al. illustrated that OCR is both age and sex-dependent (Hyer et al., 2022). Through multiple studies, our lab has established that a HFD decreases mitochondrial OCR in synaptosomes from male brains and increases mitochondrial OCR in synaptosomes of female brains (Kloster et al., 2021). However, the extent to which this finding is brain regions-specific and how diet may precipitate age-related decline remains unknown. An interesting area of research highlights the importance of estrogen in relation to mitochondrial function. Mitochondria initiate steroidogenesis, and estrogen has been shown to act through mitochondrial mechanisms to hinder the effects of oxidative stress (Gaignard et al., 2015). Therefore, estrogen could provide answers as to why females seem to be protected from cognitive decline.

**Sex and estrogen as biological variables**

The field of research is largely dominated by studies conducted on male subjects with very little focus on females. This is due to the presence of estrus cycles in female research animals that some believe serve as confounding factors when conducting research. Nonetheless, in the scope of the present study, there have been several
publications put forth that address the effects of a high fructose diet on multiple aspects of various organisms, yet the majority of studies have been conducted on male subjects only. Females are equally susceptible to the metabolic disturbances and pathophysiological outcomes associated with fructose-induced metabolic syndrome, and the downstream diseases as a result. Puberty marks the most profound shift in the endocrine system, magnifying sex differences in relation to insulin sensitivity, hypertension, and lipid levels (Vasudevan et al., 2005). Recently, research from our group has indicated that while males are more susceptible to cognitive rigidity and mitochondrial dysfunction, females tend to demonstrate more physiologic perturbations, and seem to be protected from cognitive decline (Kloster et al., 2021; Hyer et al., 2018), at least until menopause (Cournot et al., 2006). Interestingly, women are twice as likely to experience depression and anxiety in their lifetime when compared to men, a trend that emerges after puberty (Ford & Erlinger, 2004). While increased fructose consumption seems to impair the sexes differentially, the interaction between diet and sex, in the context of aging, remains largely unknown.

An additional factor to consider when addressing the sexes, is the presence of sex steroids, particularly those that interact with certain neuronal processes. Increasing amounts of research suggest that estrogen, and therefore estradiol in rats, plays a neuroprotective role in regards to cognitive decline, homeostatic challenges, and metabolic syndrome (Arevalo et al., 2014; Carswell et al., 2000). Recent studies from our lab have shown similar results – males being susceptible to cognitive rigidity following fructose consumption, while females remain largely unaffected (Hyer et al., 2018). This is likely due to estrogen’s interaction with mitochondrial mechanisms, as
mentioned above, through which the hormone acts to hinder excessive ROS production, regulate calcium loading, and preserve the mitochondrial membrane during times of stress (Wang et al., 2001; Nilsen, 2008). While the set of pathways through which estrogen operates are not fully understood, hormone replacement therapy (HRT) is associated with a significant reduction in insulin resistance, a decrease in hyperinsulinism, a reduction in visceral fat, an improvement in body composition, better control of arterial hypertension and a diminished risk of developing AD (Riant et al., 2009; Fillit, 2002). In vitro work from a previous study showed that administration of estradiol to hippocampal neurons inactivates caspase 3, a protease involved in apoptosis and cleavage of tau. Cleavage of tau results in the formation of neurofibrillary tangles, which together with the formation of amyloid-beta plaques, acts as the hallmarks for AD (Maitner, 2018).

Both sex and estrogen serve as biological variables in the context of metabolic syndrome, mitochondrial dysfunction, and cognitive decline. Given the abundance of literature addressing the effects of a HFD on male rats, it is logical to perform this study on females to better elucidate the differential outcomes on physiology, behavior, cognition, and mitochondrial function. Additionally, this study takes age into consideration as a factor for the interaction of diet and sex in the context of aging.

I hypothesized that fructose consumption at varied levels beginning in adulthood would result in physiological disturbances, altered metabolic function, and reduced cognitive flexibility of aged female rats. Further, I hypothesized that OVX would impact female rats increasing their susceptibility to the deleterious effects of fructose ingestion, similar to what has been seen in males. Following a description of the materials and
methods utilized, I will outline the data collected from this study and discuss the implications of the results.
Chapter Two

Materials and Methods

Animal Husbandry

Retired breeder female Wistar rats (n=30) 270-365 PND were procured from Charles River (Morrisville, N.C.) and assigned to experimental group 1. Retired breeder female Wistar rats (n=7) on site were incorporated into experimental group 2 along with female Wistar rats (n=6) from a previous study. 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. Experimental group 1 was paired and cage mates were assigned either a control chow diet (n=10); an 18% fructose diet (18FD) (n=10); or a 55% fructose diet (55FD) (n=10) after 7 days in our facility. Experimental group 2 underwent ovariectomies (n=6) and sham surgery (n=7). A week after surgery, rats were paired and all were assigned a high fructose diet (55FD) (n=13). All studies were conducted in accordance with the 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
Rats were either maintained on a control chow diet, or assigned a medium 18% fructose diet (18FD), or a high 55% fructose diet (55FD) and remained on the assigned diet until the conclusion of the experiment at 12 weeks. The chow diet was comprised of the Envigo Lab Diet 7012 (Teklad LM-485); the 18% fructose diet (18FD) consisted of 13% kcal fat, 23% kcal protein, and 18% kcal fructose (Research Diets D21063002); and the 55% fructose diet (55FD) was 10% kcal fat, 19% kcal protein, and 55% kcal fructose (Research Diets D050111802). Food consumption was tracked throughout the study by weighing the remaining food in the cage twice weekly before replacing the food with 300 g of new food. Cages were checked for food that had fallen to the bottom of the cage and all rats received water ad libitum. Additionally, body weight was recorded and tracked twice weekly on days when food consumption was measured. From these data, a weekly average weight and a weekly average consumption was calculated and used to determine caloric efficiency (milligram weight gained per kilocalorie consumed). Since consumption was tracked per cage, it was assumed that each rat ate 50% of the amount consumed and therefore this measure is imprecise. It does, however, highlight metabolic changes as a result of diet.

Blood Glucose Testing

Blood glucose levels were obtained from experimental subjects at 3 times over the course of the experiment: initial measure before starting the diet, approximately halfway through the 12 weeks on the diet (prior to behavioral testing), and end of dietary
week 12 (upon rapid decapitation). To accomplish this, rats were humanely restrained by bundling them in a bench pad leaving their tail exposed. A tail prick was done in the lateral tail vein using a sterile 25 gauge needle and a Freestyle Glucometer with Freestyle Lite test strips (Abbott Diabetes Care Inc., Alameda, CA) was used to obtain the reading.

Ovariectomy

Ovariectomies were performed solely on experimental group 2 before assigning them to their diet. 6 rats underwent ovariectomies and 7 rats underwent sham surgeries to avoid confounding variables. Rats were sedated using a mixture of 40 mg/mL Ketamine, 8 mg/mL Xylazine, 4 mg/mL Acepromazine dissolved in Saline. Amount administered was calculated using body weight and was injected intraperitoneally. Upon sedation, rats were administered 5 mg/mL subcutaneous Meloxicam (Covetrus, 11695-6936-1) to act as an anti-inflammatory. Rats were then shaved on either side above their thigh area and a flexible rectal probe (Harvard Apparatus, 1.6 mm O.D., 55-7021) was inserted to record internal temperature. Shaved areas were cleaned with alternating betadine solution (povidone-iodine, 5%, 301018-OC) and 70% ethanol to ensure sterility and some rats were administered isoflurane (Matrx, 0706VAP10138) depending on the effects of the Ketamine. To reduce hypothermia, rats were placed on a sterile bench pad atop a heating pad to keep their core temperature from dropping. Surgery consisted of cutting through the abdominal wall, musculature, and locating the
ovary, then tying sterile, dissolvable suture (Ethicon, J464G Suture 4-0 Coated Vicryl 18” VLT Braided P-3) around the fallopian tube before excising the ovary using surgical scissors. After ensuring all of the ovary was removed and eliminating all bleeding, the musculature was sutured closed along with the abdominal wall using dissolvable suture, before stapling the external wound closed. For sham animals, the procedure was repeated without tying the fallopian tube or excising the ovary. Post-surgery, all animals were placed on a homeothermic pad (Harvard Apparatus, 55-7022) and monitored until waking. All rats were monitored daily for 7 days post-operative and staples were removed on day 7.

**Vaginal Cytology**

Vaginal cytology was used to assess estrus cycle stage in the female rats over a 5-day period 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 μL of phosphate buffer solution (PBS) 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, females that had undergone ovariectomies were similarly wrapped in a bench pad and had their vaginal canal exposed, yet no flushing was done so as to not irritate the area.
**Behavioral Testing**

Behavioral testing was conducted at the 10 week point of the subjects’ designated diet for 12 full weeks and was conducted just prior to collections. Behavioral testing consisted of the Barnes Maze Task and was exclusively conducted on the first experimental group. All rats were habituated to the behavioral test suite for 4 days prior to testing. Behavioral testing was conducted in the middle of the rats’ light cycle and at ~2,500 lux. Barnes Maze was video recorded and tracked using Ethovision XT (Noldus Information Technologies; Leesburg, VA).

**Barnes Maze**

Spatial learning and memory were assessed using the Barnes Maze (protocol adapted from Sunyer et al., 2007). Testing occurred in a neutral room illuminated to approximately 2500 lux on a black, circular table (121 cm in diameter) elevated 91.5 cm from the floor with 20 equidistant holes (10 cm in diameter) around the perimeter fitted with 19 false boxes and one goal box. Placement of the goal box was counterbalanced across rats. Prior to testing, rats were habituated to the room for 30 min a day for 4 days. All trials were recorded with an overhead camera and tracked using EthoVisionXT 13.0 (Noldus, Leesburg, VA). Acquisition training occurred over five consecutive days where rats were placed in the center of the maze in a start box and given 3 min to locate the goal box. Immediately after the rat entered the goal box, the experimenter turned off the lights and covered the entry into the goal box for 2 min before a 4-min inter-trial interval in the home cage followed by a second trial. The maze was cleaned.
with 70% ethanol between every trial. Dependent variables included latency to the goal box, velocity, error rate (defined as the number of non-goal box visits prior to locating the goal box), and time spent in the periphery which served as a proxy for search strategy. A single 5-min memory probe took place 72h following the final acquisition day. During probe testing, the goal box was replaced with a false box allowing for quantification of latency to locate the goal box location and number of revisits to the goal box location over a 5-min trial. Reversal learning began 24 h after the probe assessment to measure cognitive flexibility and was identical to that of acquisition learning with the goal box placed in the opposite quadrant (180 degrees) from its initial location. Training occurred over three days. A reversal probe assessment was completed 72h following the final reversal day to assess memory for the new and old goal box locations.

_Tissue Collection_

24 hours after the conclusion of behavioral testing, tissue was collected from experimental group 1. Following rapid decapitation, trunk blood was collected and the uterus was extracted, weighed, flash frozen and stored at -80°C. Brains were removed from subjects and bisected. The left hemisphere was dissected and the amygdala, caudate putamen, cerebellum, hippocampus, hypothalamus, nucleus accumbens, and prefrontal cortex were flash frozen and stored for later analysis (not included in this
thesis document). The right hemisphere was utilized for a cell mitochondrial stress test in the Seahorse XT Analyzer (Agilent).

**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 and bisected. From the left hemisphere, seven areas were removed and flash frozen for later analysis, while 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. The prefrontal cortex and hippocampus were removed from the right hemisphere and analyzed separately using Seahorse XT Analyzer. The prefrontal cortex and hippocampus were separately 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 6 strokes with first the loose then tight plunger, respectively. The homogenate was centrifuged (Eppendorf 5810R) at 3600 rpm for 10 minutes at 4°C. The supernatant was removed and 3.85 mL was layered on top of a discontinuous Percoll gradient (4 ml layers of 23, 15, 10, 3% Percoll in homogenization buffer and a 1 mL layer of pure homogenization buffer) in a 26 mL centrifuge tube and spun at 32,500g 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 Na₂HPO₄, 1 mM MgCl₂, 5 mM NaHCO₃, 5 mM KCl, 140 mM NaCl, pH 7.4), and centrifuged at 15,000g 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 using Nanodrop (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 warmed 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). 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).

**Estradiol ELISA**

Estradiol assessment was adapted from Shaw et al., (2021). To confirm the lack of cycling estradiol in the ovariectomized group of the second experimental group, an Enzyme-Linked Immunosorbent Assay (ELISA) was performed for each sample. Serum concentrations of 17β-estradiol (sensitivity 14pg/mL, Cat # ADI-901–174, Enzo Life Sciences) were assessed according to manufacturer’s instructions. Samples underwent a liquid-liquid extraction; 250 uL of serum was mixed with 2 mL of ethyl ether (Cat # EX0185-8, Millipore Sigma, Burlington, MA, USA), vortexed, and placed in a bath of dry ice and methanol to solidify the inorganic phase. The organic phase was decanted into a glass test tube and placed in a 37°C dry bath for one hour to allow evaporation of the residual ethyl ether. Extractions were repeated twice per sample and then reconstituted with 240 uL of Assay Buffer and run undiluted.

**Statistical Analyses**
Data were analyzed using GraphPad Prism Software (San Diego, CA). 1-way and 2-way Analyses of Variance (ANOVA) were run in order to compare endpoints with multiple time points per subject – particularly weight, food consumption, glucose, Barnes Maze, and mitochondrial respiration. Sidak’s multiple comparisons tests, Kruskal-Wallis tests, and Mann-Whitney tests were used to verify the results of the ANOVAs. A t-test was run to assess circulating estradiol using ELISA techniques. Group sizes reflect statistical power considerations as determined by power tests where preliminary data on variability exist, with $\alpha=0.05$. 
Chapter Three

Results

Physiology is altered in aged female rats on a fructose diet

Examining individual weights per week, a 2-way ANOVA showed a main effect of week ($F_{(3.333,90.00)}=69.15; p<0.0001$), diet ($F_{(2,27)}=5.736; p=0.0084$), and subject ($F_{(27,297)}=258.9; p<0.0001$), as well as significant interactions between week and diet ($F_{(22,297)}=4.197; p<0.0001$) such that females on a 18FD had significantly higher body weights than their counterparts. There was no significant difference between the 55FD and chow control rats (Figure 1A).

To assess the effect of diet on weight over time, an individual 2-way ANOVA was conducted examining the change in weight from baseline per rat. A main effect of week ($F_{(3.333,90.00)}=69.15; p<0.0001$), diet ($F_{(2,27)}=9.416; p=0.0008$), and subject ($F_{(27,297)}=44.27; p<0.0001$), as well as significant interactions between week and diet ($F_{(22,297)}=4.197; p<0.0001$) was concluded indicating that aged females on a 18FD gained significantly more weight week for week when compared to 55FD and chow groups (Figure 1B). All rats, regardless of diet, gained weight throughout the study.

Using a 2-way ANOVA, food consumption was analyzed and displayed a main effect of week ($F_{(3.648,98.49)}=27.37; p<0.0001$), diet ($F_{(2,27)}=24.90; p<0.0001$), and subject ($F_{(27,270)}=5.855; p<0.0001$), as well as significant interactions between week and diet ($F_{(20,270)}=7.005; p<0.0001$) such that starting at week 4, rats on a 55FD consumed
significantly less than chow rats, and starting week 5, rats of a 18FD consumed significantly less than the chow group (Figure 2A). There was no significant difference among the 55FD and 18FD groups.

Caloric efficiency was calculated by dividing weight gained per day by the estimated amount of calories consumed per day (daily values calculated from weekly values divided by 7). Caloric consumption was estimated from the grams of food consumed per animal multiplied by the known caloric content of the respective diet (3.35 kcal/gram of chow; 9.91 kcal/gram of 18FD; 3.85 kcal/gram of 55FD). A 2-way ANOVA displayed a main effect of week ($F_{(4,580,123.7)}=3.901; p=0.0034$) and diet ($F_{(2,27)}=5.137; p=0.0129$), but no significant interaction between the two (Figure 2B).

2-way ANOVA revealed no significant effect of diet on circulating blood glucose levels (p>0.05).

Physiology is unchanged in ovariectomized rats on a 55FD

Similar analyses were conducted on the second experimental group. A 2-way ANOVA of weights per week found a main effect of week ($F_{(1,933,21.26)}=81.10; p<0.0001$) and subject ($F_{(11,121)}=246.3; p<0.0001$) but no significance of OVX or interaction between OVX and week (p>0.05) (Figure 3A).

Further analysis of change in weight gained per week revealed similar results to the 2-way ANOVA described above. A main effect of week ($F_{(1,933,21.26)}=81.10; p<0.0001$) and subject ($F_{(11,121)}=63.61; p<0.0001$) was displayed, but no significance of OVX or interaction between OVX and week (p>0.05) (Figure 3B).
A 2-way ANOVA of food consumption displayed a main effect of week \((F_{(2.807,30.88)}=15.52; p<0.0001)\), OVX \((F_{(1,11)}=7.626; p=0.0185)\), and subject \((F_{(11,121)}=7.765; p<0.0001)\), as well as significant interactions between week and OVX \((F_{(11,121)}=4.168; p<0.0001)\). Sidak’s multiple comparisons test revealed that this interaction was driven by week 11 \((p=0.0001)\) (Figure 4A).

Caloric efficiency was calculated as described above, utilizing the known caloric content value of 3.85. There was no significance of estradiol or subject, but a main effect of week \((F_{(5.554,61.09)}=7.855; p<0.0001)\) and an interaction of estradiol and week \((F_{(11,121)}=1.985; p=0.0355)\) was observed (Figure 4B).

Unpaired t-tests revealed no significant effect of estradiol on circulating blood glucose levels \((p>0.05)\) (Table 1).

*Fructose consumption does not alter acquisition learning for female rats*

During initial acquisition on the Barnes Maze assessment, all rats successfully located the goal box with increased accuracy as indicated by the decrease in latency (Figure 5A) across the five days \((F_{(2.439,65.85)}=20.14; p<0.0001)\) along with a decrease in errors \((F_{(3.049,82.33)}=7.515; p=0.0002)\) (Figure 5B). There was no significant effect of diet on latency, errors, velocity, or distance, and there was no significant interaction between training day and diet for acquisition learning \((p>0.05)\).
Reversal learning is not impacted by fructose consumption in females

Regardless of diet group, aged female rats were unable to learn the reversal task over the course of the three days. There was no significant effect of day or diet and no interaction between the two in regards to latency (Figure 5D) or errors made (p>0.05) (Figure 5E). Velocity, however, was significantly altered across reversal learning ($F_{(1.952,52.70)}$=10.63; p=0.0001) showing that the females got progressively faster at locating the new goal box (Figure 5F). These data suggest that all groups showed cognitive rigidity.

Memory is not altered by fructose consumption

To examine the role of memory, 1-way ANOVAs were run on the primary errors from probes 1 and 2. Probe 1 revealed a p value greater than 0.05 (p=0.6446) indicating that there was no significant difference between the groups in the amount of errors made when locating the goal box (Figure 6A). Probe 2 displayed a more progressive trend where the 55FD group made the most primary errors followed by the 18FD, then the chow group when locating the new goal box (Figure 6B). However, the P-value was still not of significance at 0.3432. Probe 2 was further analyzed to uncover if there was a difference between the duration of time spent in the quadrant with the original goal box (acquisition) versus the duration of time spent in the quadrant with the new target.
Fructose consumption alters oxygen consumption rate in a region-specific manner

Within the hippocampal region, there was a main effect of measurement ($F_{(1.009,19.16)}=8.997; p=0.0072$), subject ($F_{(19,209)}=21.45; p<0.0001$), and an interaction of measurement and diet ($F_{(22,209)}=1.898; p=0.0113$) (Figure 7A) such that a 18FD had significantly higher levels of respiration when compared with 55FD and control. Individually, diet did not have a significant effect on OCR in the hippocampus ($p>0.05$). Additionally, the 2-way ANOVA done on the prefrontal cortex displayed a main effect of measurement ($F_{(1.096,26.30)}=68.77; p<0.0001$) and subject ($F_{(24,264)}=19.44; p<0.0001$), but no significant effects of diet or interaction between measurement and diet ($p>0.05$) (Figure 7B).

Ovariectomy does not alter the effects of a 55FD on oxygen consumption rate of synaptosomal mitochondria

A 2-way ANOVA conducted on the hippocampus of OVX and non-OVX females displayed a main effect of measurement ($F_{(1.151,9.206)}=84.38; p<0.0001$) and subject ($F_{(8,88)}=24.11; p<0.0001$), but did not demonstrate an interaction between measurement and OVX ($p>0.05$) (Figure 8A). Analysis of the prefrontal cortex revealed similar results,
generating a main effect of measurement \((F_{(1,090,11.99)}=84.38; p<0.0001)\) and subject \((F_{(11,121)}=24.11; p<0.0001)\), but no significant interaction or individual effect of OVX \((p>0.05)\) (Figure 8B).

*Lower levels of circulating estradiol was confirmed in the OVX group*

An unpaired t-test was conducted to assess circulating levels of estradiol in the second experimental group. Results revealed significantly higher levels of circulating estradiol in the non-OVX group when compared with the OVX females \((p=0.0191)\) (Figure 9).
Figure 1: *Physiology is altered in older female rats on a fructose diet.* A) Female rats fed an 18FD weighed significantly more week to week than 55FD and chow counterparts. Symbols represent mean ± SEM. *p<0.05. B) Change in weight from baseline week to week was significantly higher in the 18FD group when compared with 55FD and chow. All rats gained weight throughout the 12 weeks. Symbols represent mean ± SEM. *p<0.05.
Figure 2: *Physiology is altered in older female rats on a fructose diet.* A) The chow group consumed significantly more food than both fructose groups starting at week 4 and continuing throughout the duration of the diet. Symbols represent mean ± SEM. *p<0.05. B) There were no significant alterations in caloric efficiency in any diet group. Symbols represent mean ± SEM. *p<0.05.
Figure 3: *Physiology is unchanged in ovariectomized rats on a 55FD.* A) There was no significant difference observed between the OVX and non-OVX groups in relation to weekly weight gain. All rats gained weight while on the diet. Symbols represent mean ± SEM. *p<0.05. B) No significant difference between the groups in weight gain was observed. Symbols represent mean ± SEM. *p<0.05.
Figure 4: Physiology is unchanged in ovariectomized rats on a 55FD. A) A significant difference between week and estradiol was observed during week 11 only. Symbols represent mean ± SEM. *p<0.05. B) There was no significant effect of estradiol on caloric efficiency while on a 55FD. Symbols represent mean ± SEM. *p<0.05.
Figure 5: Effects of fructose consumption on acquisition and reversal learning. A) All rats displayed decreased latency to locate the goal box across acquisition. B) Summed errors decreased across the 5 days. C) Average velocity increased meaning that the rats got progressively faster at the task indicating learning. D) Latency to locate the new goal box did not decrease significantly across all groups. E) Summed errors decreased, then increased for the chow group on day 3. F) Average velocity increased across reversal indicating that the rats got progressively faster at the task. Symbols represent mean ± SEM. *p<0.05.
Figure 6: Memory is not altered by fructose consumption. A) No significant difference between groups was established in probe 1. Symbols represent mean ± SEM. *p<0.05. B) Probe 2 revealed an upward trend with the 55FD group tending to make the most primary errors, but no significance was established. Symbols represent mean ± SEM. *p<0.05.
Figure 7: Fructose consumption alters oxygen consumption rate in a region-specific manner. A) The 18% fructose group showed higher levels of OCR than its counterparts in the hippocampal region. Symbols represent mean ± SEM. *p<0.05. B) There was no significant difference between groups in the prefrontal cortex. Symbols represent mean ± SEM. *p<0.05.
Figure 8: Ovariectomy does not alter the effects of a 55FD on oxygen consumption rate of synaptosomal mitochondria A) No significant difference in OCR was observed between OVX and non-OVX groups in the hippocampus. Symbols represent mean ± SEM. *p<0.05. B) No significant difference in OCR between groups in the prefrontal cortex. Symbols represent mean ± SEM. *p<0.05.
**Figure 9**  
*Ovariectomy significantly lowers circulating estradiol.* There is a significant difference between the non-OVX and OVX animals in means circulating estradiol levels. Non-OVX showed significantly higher levels of circulating estradiol, as expected. Symbols represent mean ± SEM. *p<0.05.
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**Table 1:** Physiology is unchanged in ovariectomized rats on a 55FD. Circulating blood glucose levels are indicated per group by week. There was no significant change by measurement, indicated by the P values. Symbols represent mean ± SEM.
Chapter Four

Discussion

Due to the accelerated rate of fructose consumption in recent years, particular focus has been placed on researching implications of consuming such a diet in relation to physiology, biology, as well as neurology. Numerous studies have identified fructose as an instigator of metabolic syndrome (Rutledge & Adeli, 2008), and seen the effects of a diet high in fructose in male, and to some extent, female rats. Some of the most deleterious effects reported are cognitive decline, mitochondrial dysfunction, and physiological alterations (Kloster et al., 2021). However, estrogen seems to serve as a protective factor for cognitive-related decline in females (Arevalo et al., 2014). The findings of this study display that consuming varied percentages of fructose diets have differential impacts on the physiology, behavior & cognition, and mitochondrial function of female rats while also uncovering that estradiol may not serve as a protective factor for cognitive decline.

*Fructose consumption altered select physiological parameters in female rats*

The consumption of diets with varied levels of fructose altered the physiological parameters of one-year-old (aged) female Wistar rats on the 18FD, but not those on the 55FD. While rats on all diets gained weight throughout the study, those on the 18FD gained significantly more weight than their counterparts, including the 55FD group. Additionally, rats on the control chow diet consumed significantly more than the 55FD group starting in week 4, as well as the 18FD starting week 5. The 18FD group gaining
significantly more weight while consuming significantly less than the control group indicates a diet-induced effect of fructose, specifically the 18FD. Diets high in fructose have been shown to increase levels of circulating plasma leptin, the satiety hormone, and eventually lead to leptin resistance. In a previous study by Shapiro et al., Sprague-Dawley rats were placed either on fructose-free or a high-fructose diet for 6 months, and then tested for leptin resistance. Results indicated that those on the HFD showed increased levels of leptin and decreased levels of food consumption which may serve as the reason for decreased consumption on the 55FD and 18FD. Additionally, the study concluded that fructose-induced leptin resistance accelerates high-fat induced obesity (Shapiro et al., 2008). This has been a consistent finding in our lab.

Upon analysis of caloric efficiency, there was no significant difference apparent between any of the groups indicating that diet alone, and not energy utilization, is a possible source of the observed increase in body mass of the 18FD. Previous literature has analyzed the effects of a fructose-rich diet and seen significant effects in females when compared to males. Particularly, female rats have been characterized by decreased glycemia, increased triglycerides, enlarged visceral adipose tissue and increased absolute mass of liver, without changes in systolic blood pressure and insulin sensitivity (Koricanac et al., 2013). In addition, our lab has extensively researched the physiological alterations of a HFD on males and females and has conclusively demonstrated that females tend to gain more weight than males without altering caloric efficiency (Carswell et al., 2000).
**Presence of estradiol does not significantly alter physiology**

A 55FD impacted the physiological parameters of ovariectomized (OVX) and non-ovariectomized rats equivalently. Similarly to the first experimental group, all rats on the 55FD, regardless of OVX status, gained weight throughout the study, likely due to natural weight gain associated with aging. There was, however, no significant difference in body mass gained between the two groups. Additionally, analysis of food consumption revealed no significant difference in the amount consumed over the duration of the study and caloric efficiency was not significant indicating no differential utilization of energy. These findings are not consistent with previous studies which have shown that OVX rats tend to consume more and gain weight more rapidly than non-OVX rats (Roesch, 2006). Another recent study looked at weight gain, among other factors, in OVX and sham rats fed a high-fat high-fructose diet (HFFD) for 12 weeks. Results indicated that OVX rats on a HFFD gained significantly more weight than sham rats also fed the HFFD, as well as displayed central obesity, glucose intolerance, and insulin resistance (Chansela et al., 2022).

A possible explanation for the discrepancy seen in the current study is that the 55FD fed to the rats was not also high in fat. It is possible that the outcomes seen in the Chansela et al. study are due to an interaction of high fat and high fructose causing differential weight gain among OVX and sham groups. Additionally, our lab has previously uncovered that female rats tend to show increased body mass on a HFD compared to chow specifically when starting the diet during adolescence (Hyer et al., 2019). The age of the rats and the introduction of the 55FD during adulthood could therefore also account for the lack of differential weight gain in this study. In a future
cohort, it could be interesting to explore the difference in body mass following OVX and sham on a 12 week 18FD, since the present study has uncovered a tendency for females to gain more weight on a 18FD compared to a 55FD. The introduction of a medium-fructose medium-fat or even medium-fructose high-fat diet could also yield interesting results in a future study.

**Cognitive flexibility following a fructose diet**

Cognitive function was assessed on the first experimental group using the Barnes Maze Task to uncover the relationship between a 55FD, 18FD, and a control chow diet on the flexibility of memory. As mentioned previously, all rats regardless of diet, were able to learn the initial task of locating the goal box shown by a decrease in latency and additional decrease in errors. During reversal learning, however, none of the groups displayed cognitive flexibility. While the general trend of the data suggest that latency and errors did decrease over the three-day period indicating some learning, the 55FD group showed a small increase in latency on day 2. Nonetheless, none of the data significantly indicated that learning had taken place in any group and, as such, implies that all females displayed cognitive rigidity. The data collected from the probes indicate that memory is not affected by fructose. There was no significant difference among groups during probe 1 displaying that females exposed to fructose were still able to learn where the original goal box was and remember it upon recall. Probe 2, however, showed a different trend where there were generally more primary errors made by the 55FD group, followed by the 18FD group, then the control chow group. While the difference was not statistically significant, this increase in primary errors seen in groups
exposed to fructose indicates that the age of the rats may leave them more susceptible to the deleterious effects of fructose on memory and recall. Probe 2 was further analyzed to assess if time spent near the original goal box learned during acquisition differed significantly from time spent near the new goal box learned during reversal. Results revealed no significant differences in time spent near new or old targets for any diet group. This indicates that all groups did not have a preference for either goal box, but spent equal time across the maze during probe 2.

These findings are unlike those we have previously uncovered in our lab, where generally females seem to be protected from fructose-induced decline by remaining cognitively flexible (Hyer et al., 2018). Previous literature has suggested estrogen, and consequently estradiol, acts as a protective factor from cognitive decline (Arevalo et al., 2014), which serves as the motivation for the creation of the second experimental group. Since cognitive rigidity was seen in all dietary groups alike, including the control group, it is likely that these findings have to do with the interaction of age and the duration of reversal learning. Aging alone is a risk factor for cognitive decline (Legdeur et al., 2018) and while literature has previously uncovered the three-day period as being sufficient for reversal learning (Sunyer et al., 2007), it is possible that due to the age of this group of rats, an additional day would have displayed some significant cognitive flexibility, in the control group at the least. Neurological events such as neuroinflammation, altered intracellular signaling & gene expression, as well as neurogenesis & synaptic plasticity are largely thought to be associated with age-related cognitive decline (Bettio et al., 2017). Taken together, these findings indicate that the
cognitive rigidity seen in all dietary groups is likely due to aging and the cognitive decline associated with it.

\textit{Diet alters oxygen consumption rates of synaptosomal mitochondria}

Previous work from our lab has established that a diet high in fructose increases mitochondrial OCR in female brains, yet these analyses were done using whole-brain extractions (Kloster et al., 2021). For the purpose of uncovering region-specific and age-related changes, the prefrontal cortex and the hippocampus were individually extracted and analyzed using Seahorse. Consistent with previous findings, both 18FD and 55FD groups showed an increase in OCR in the hippocampus when compared with controls. While the 55FD group’s increase was not significantly different, the 18FD diet displayed a largely significant increase compared to both control and 55FD. In the prefrontal cortex, however, results yielded no significant difference between the groups, yet the 18FD group appeared increased compared to chow throughout. Interestingly, the 55FD displayed a decrease in OCR when compared to controls, an observation that was contradictory to our previous findings.

The increase seen in OCR in female rats is largely explained by sex differences between male and female rats, particularly in the light of steroidogenesis. Mitochondria play a role in initiating steroidogenesis and previous work has outlined the extent to which synaptosomal mitochondrial function is regulated by sex hormones (Gaignard et al., 2015). As mentioned previously, estrogen (and thus estradiol) offers a neuroprotective factor, particularly in the context of homeostatic challenges and metabolic syndrome (Arevalo et al., 2014; Carswell et al., 2000). Specifically, estrogen
works through mitochondrial mechanisms to hinder excessive reactive oxygen species (ROS), regulate mitochondrial calcium loading, and preserve the integrity of the mitochondrial membrane during times of stress (Wang et al., 2001; Nilsen, 2008). Therefore, the increase in OCR seen in females on a 18FD, and to some extent 55FD, may be attributed to the neuroprotection of estradiol in the synaptosomal mitochondria.

The reduced effects seen in the prefrontal cortex are consistent with previous studies. The hippocampus exhibits a higher propensity to metabolic insults, with a higher oxidative damage to proteins concomitant to a decreased catalase activity, when compared with the prefrontal cortex (Crescenzo et al., 2019). Thus, the prefrontal cortex in itself largely contributes to cognitive flexibility and spatial memory, yet it is less of a target in the analysis of synaptosomal mitochondria due to its resistant nature to the impacts of a diet medium to high in fructose.

**Estradiol does not alter oxygen consumption rates of synaptosomal mitochondria**

The basis for the creation of the second experimental group was to investigate the previously described protective effects of estrogen (estradiol) from the deleterious consequences of consuming a 55FD. As mentioned, estrogen has been shown to provide certain neuroprotective effects against metabolic syndrome and homeostatic challenges (Arevalo et al., 2014; Carswell et al., 2000), and should presumably demonstrate an increase in OCR of the non-ovariectomized group. Upon ovariectomy, rats were given 3 weeks to fully recover and to ensure that all circulating sex steroids were diminished before beginning the diet and physiological measurements. Concentration of circulating estradiol was confirmed using ELISA techniques. The
ELISA revealed significantly lower levels of circulating estradiol in the OVX group when compared with the non-OVX group, indicating that the ovariectomy procedure was successful at removing the ovaries in their entirety.

By analyzing the OCR of synaptosomal mitochondria in both the hippocampus and the prefrontal cortex of the right hemisphere, it could be determined that there was no significant protection in the non-OVX group from the effects of the 55FD. While our lab has uncovered overall sex differences in OCR, the way in which diet may precipitate age-related decline remains unknown. In a recent study of OCR and aging, Hyer et al. (Hyer et al., 2022) demonstrated age- and sex-specific alterations in OCR of the hippocampus and prefrontal cortex. Results yielded that in females, OCR increased significantly for 12-month-old (aged) rats, while it decreased in 6-month-old (mid-aged) rats when compared to 3-month-old (young) females (Hyer et al., 2022). Since these rats began the 55FD in adulthood, as opposed to the previous rats who started in adolescence, it is possible that age could play a factor. As observed in the 55FD of the first experimental group, there was no significant difference of OCR between the 55FD and chow diet groups in the hippocampus or prefrontal cortex, offering further evidence that initiation of diet in adulthood could protect against the mitochondrial disruption that comes with a 55FD. Additional work from our group has demonstrated that the negative effects of a HFD are dependent on adolescent consumption compared to beginning the diet in adulthood (Harrell et al., 2015).
Future research and conclusions

Further investigation is necessary to fully conceptualize the implications of the data presented in this study. To confirm physiological alterations between dietary groups, remaining serum samples should be examined for elevated levels of triglycerides and leptin and decreased levels of cholesterol. The leptin hormone produces the sensation of satiety (Teff et al., 2004) and recent studies have reported that fructose diets increase fasting plasma levels of leptin in rats (Mooradian et al., 2000). This ultimately leads to leptin resistance and eventual changes in energy metabolism (Abdulla et al., 2011). These studies support evident differences in energy metabolism among fructose groups, and would therefore be an interesting topic to consider for future research. Additionally, estradiol levels of the first experimental group should be assessed in order to determine any connectivity between estradiol and age as factors. Multiple publications have revealed that the age at which a diet rich in fructose is initiated has downstream effects on metabolic syndrome and energy metabolism (Huynh et al., 2008).

Additionally, analysis of OCR in synaptosomal mitochondria was performed for both experimental groups using the right hemisphere’s hippocampus and prefrontal cortex, further analysis of OCR in additional brain regions from the left hemisphere should be done. Other brain regions could provide more insights regarding the natural fluctuation of OCR for various brain regions as well as display how diet, estradiol, and/or age impacts those different fluctuations. For example, a 2016 paper analyzed the mitochondrial bioenergetic parameters in five different brain regions among young, adult, aged, and old rats to understand the age-related differences in select brain
regions (Pandya et al., 2016). The results indicate age and brain region-specific patterns in mitochondrial functional endpoints, which highlights the potential to uncover a more comprehensive understanding of the natural changes in OCR seen with age, in addition to diet and estradiol.

Taken together, a future area of research involves interpreting the interaction of when the diet is started and the duration of time spent on the diet. As mentioned, the age of diet onset plays a significant role in the negative impacts of a fructose diet. de Moura et al. noted that younger animals tend to have some protective mechanisms against fructose-induced metabolic syndrome, yet this was noted solely among a HFD group of animals and not a medium-fructose diet (MFD) (de Moura et al., 2008). Multiple sex differences have also been revealed within our lab for rats beginning a HFD in adolescence (Kloster et al., 2021; Hyer et al., 2018; Hyer et al., 2019), yet the consequences of a long-term HFD or MFD remain largely unknown. A study by Abdullah et al. saw significantly higher blood pressure, circulating insulin, total cholesterol, and triglyceride levels in a high fructose group of Sprague-Dawley rats, yet the diet was only 20 weeks (Abdullah et al., 2009), similar to most literature. Future studies should uncover the effects of varied levels of fructose diets started in adolescence and continued beyond 20 weeks.

There is substantial evidence that adolescent rats versus aged rats tend to display altered levels of oxygen consumption in the synaptosomal mitochondria of the hippocampus and prefrontal cortex. Within our lab, data on female rats have displayed that aged rats tend to show lower OCR compared to younger rats, while middle aged rats have revealed elevated OCR (Hyer et al., 2022). Hyer et al. further uncovered the
differences seen in male rats. These sex and age dependent mitochondrial effects have been amplified by Guevara et al. – stating that females tend to show more differentiated mitochondria than males (Guevara et al., 2009). Finally, Kloster et al. highlighted the apparent sex differences in OCR of male and female rats on a HFD started in adolescence (Kloster et al., 2021) solidifying the need for an experimental group consisting of males and females, started in adolescence, with varied levels of fructose.

Importantly, one of the questions this study aimed to address was if the reported neuroprotective effects of estrogen and estradiol (Arevalo et al., 2014) would be evident in the OCR data collected from OVX and non-OVX rats on a 55FD for 12 weeks. Outlined extensively throughout this study, estrogen and its counterparts seem to offer neuroprotection from the deleterious effects of a HFD, particularly in relation to mitochondrial function and cognitive impairment. While there was no indication that estradiol protected the non-OVX rats from mitochondrial dysfunction, this study was not able to assess spatial memory and cognitive rigidity using the Barnes Maze Task as previously described (Shaw et al., 2020). Performing cognitive testing on OVX and non-OVX rats consequently serves as a future area of research. A previous study investigated the effects of estradiol or vehicle treatment on OVX or sham-operated rats by testing their performance on Barnes Maze. Results suggested that estradiol treatment improved performance both in OVX and sham rats suggesting that estrogen does play a role in cognition (Ping et al., 2008).

Additionally, the second experimental group consisted of OVX and non-OVX rats all on a 55FD. In accordance with the results of this study, future investigation of the effects seen on a 18FD as well as a control chow diet would be an interesting line of
research. As discussed above, the age of the rats and the duration of the diet are both factors that could be manipulated so that the rats could be ovariectomized earlier while starting their respective diet during adolescence and continuing beyond the 20 week mark. In contrast, the diet could start during adulthood and continue beyond 20 weeks, followed by Barnes Maze (or alternative cognitive testing) to assess the differences between starting a diet during adolescence versus adulthood and the interaction of estradiol.

In conclusion, this study represents the expansion of previous lab work on the consequences of a high fructose diet started in adolescence to include a medium fructose diet begun in aged female rats. Additionally, we investigated the question of estrogen’s protective effect on OCR in synaptosomal mitochondria of the hippocampus and prefrontal cortex. The data presented outline the extensive potential of a 18FD to induce physiological alterations as well as increased OCR in the hippocampus of aged female rats. The effects of estrogen were not significant in relation to mitochondrial respiration, however, future cognitive assessments will further address estradiol’s neuroprotective element. Both physiological parameters and mitochondrial metabolism serve as interesting areas of research to connect the biological state of an organism to its overall health outcomes.
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