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Sex Difference in Long Term High-Fat Induced Type II Diabetes in Mice: Effects on Mitochondrial Function

A thesis submitted in partial satisfaction of the requirements

for the degree Master of Science

in

Biology

By

Marianne Thio

Committee in charge:

Professor Hemal Patel, Chair
Professor Randolph Hampton, Co-Chair
Professor Katherine Petrie

2020

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University of California San Diego

2020

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ABSTRACT OF THE THESIS

Sex Difference in Long Term High-Fat Induced Type II Diabetes in Mice: Effects on Mitochondrial Function

by

Marianne Thio

Master of Science in Biology

University of California San Diego, 2020

Professor Hemal Patel, Chair
Professor Randolph Hampton, Co-Chair

As the rate of Type II Diabetes Mellitus (T2DM) develops not only in adults but in youth, many other health related problems are starting to arise in association. A major complication related to T2DM is cardiovascular disease, which female diabetic patients have been found to have a worsened outcome compared to male diabetic patients. To get a better understanding of

the sex difference in cardiovascular disease and other complications associated with T2DM, mitochondrial function was investigated to find if it was an underlying cause and if there was a difference between males and females. Through an obesity driven T2DM model, young female and male mice were fed a 45% kcal fat diet (HFD) to induce diabetes and a 10% kcal fat diet (LFD) as control. Males on HFD significantly increased weight with a more profound impairment to glucose tolerance, while females on HFD slowly increased weight, developed ulcerative dermatitis (UD), and did not have as profound impairment to glucose tolerance. There was also a statistically significant three-way interaction between mitochondrial function, gender and diet in the hippocampus and kidney. A two-way interaction between mitochondrial function and diet was found to be statistically significant in the heart, but a statistically significant interaction between mitochondrial function and gender was found in the hippocampus, liver and pancreas.

INTRODUCTION

Diabetes is a growing health concern in many countries as diabetes is associated with multiple risk factors, such as cardiovascular disease, lower-extremity amputation, hyperglycemia and hypoglycemia, that can lead to high morbidity and mortality rates [1], [2], [3],[4]. According to the CDC's National Diabetes Statistics Report 2020, 34.3 million people of all ages had diabetes with 34.1 million being adults [4]. There are currently two main types of diabetes that is known, Type I Diabetes Mellitus (T1DM) and Type II Diabetes Mellitus (T2DM). T1DM is known as the insulin-dependent diabetes, where it is a chronic autoimmune condition in which the β cells, affected by β cell autoantibodies, in the pancreas that secrete insulin is unable to produce enough or any insulin [2], [5]. While T1DM is a chronic condition that starts at birth, T2DM is caused by insulin resistance caused by unreceptive insulin receptors from irregularity in insulin signaling [6]. Although T1DM is important, it is even more important to study T2DM because it is not only currently the most common form of diabetes but there is also a higher frequency of T2DM-morbitidy and mortality in both adolescents and adults, compared to T1DM [4], [7].

Development of T2DM

In healthy individuals, β cells in the pancreas releases insulin into the blood stream when it senses a rise in the blood glucose [8]. Insulin plays an important role in regulating the suppression of glucose production and release through the prevention of glucagon production and uptake of glucose in insulin-sensitive tissue (adipose, muscle, and liver) [9]. The uptake of glucose causes glycogen production and the blood glucose level to decrease back to homeostasis [8], [9]. Contrarily, glucagon, which is produced by α cells in the pancreas, is released into the blood stream when there is a decline in blood glucose. Glucagon prevents the secretion of insulin

and release of glucose into the blood stream, allowing blood glucose levels to increase back to homeostasis [10]. Both insulin and glucagon work in a negative feedback loop to keep blood glucose levels at homeostasis [8], [9], [10].

The onset of T2DM usually starts with insulin resistance or insufficient production of insulin [11]. When someone is insulin resistant, there is a decreased amount of glucose entering cells and supplying energy, increasing blood glucose levels. Therefore, the β cells increase the production and release of insulin to maintain normal blood glucose levels [9]. The constant increase production of insulin by β cells eventually leads to post-receptor defects in insulin signaling, resulting in decreased uptake of glucose and a buildup of glucose in the blood, creating a condition called hyperglycemia [6]. The post-receptor defects can be mutations in the receptors or a decrease number of receptors, which lead to the decrease binding of insulin, or insulin resistance [12]. As a result of insulin resistance, there is a decreased output of insulin in β cells and an increase in blood glucose levels. The inverse relationship between insulin output and blood glucose levels disrupts the negative feedback loop that keeps blood glucose at homeostasis. This disruption results in constant high blood glucose and is when someone is diagnosed with T2DM [6], [9], [13].

Mitochondrial Function in T2DM

When blood glucose is high, there is insufficient glucose in the cell. Glucose is an essential molecule for cells to undergo glycolysis in the cytoplasm, which provides NADH, required by the mitochondria to create adenosine triphosphate (ATP) through oxidative phosphorylation and pyruvate for the citric acid cycle [14]. Mitochondria are crucial organelles for eukaryotic organisms, as it creates the energy, or ATP, required for the cell to function, but can also produces reactive oxygen species (ROS) and regulates apoptosis [15]. A mitochondrion

is made up of an outer membrane, an inter-membranous space, a matrix and an inner membrane, containing the electron transport chain comprised of 5 complexes: complex I, complex II, complex III, complex IV and complex V [15]. The citric acid cycle, which takes place in the mitochondrial matrix, uses pyruvate to create FADH2 and NADH. NADH from both glycolysis and the citric acid cycle donates its electrons to complex I, which FADH from the citric acid cycle donates its electrons to complex II [15], [16]. Coenzyme Q transports the electrons from complex I and complex II to complex III, and cytochrome c transports the electrons from complex III to complex IV [15]. This transportation of electrons from different complexes allows for protons to be pumped into the inter-membranous space, which creates a proton gradient. The movement of proton down the gradient provides the energy needed for ATP production in complex V [15]. However, when there is limited oxidative phosphorylation, the electrons on complex I are donated to O2, creating hydrogen peroxide (H2O2), a ROS [15]. ROS can impair proteins, lipids and encourage further production of ROS [15]. Under this cellular stress, the mitochondrial permeability transition pore can open, releasing cytochrome and apoptosis initiating factor, which ultimately induces apoptosis [15]. Therefore, when someone has T2DM and is insulin resistant, there is a decreased amount of glucose entering the cell, meaning NADH and FADH2 may not be created [14], [15]. Kwak and colleagues have found decreased oxidative phosphorylation in T2DM, but what specifically is being affected is still unknown [15].

Influencing Factors of T2DM

A major influencing factor of the development of T2DM is obesity [9]. Obesity, defined by the excess amount of adipose tissue, can be caused by overnutrition, which can lead to lipogenesis and fat storage in white adipose tissue. More specifically, an increased consumption of dietary fat can lead to accelerated weight gain from the breakdown of the dietary fat into free

fatty acids (FFA) and develop into obesity [17]. FFA is the primary substrate in the synthesis of triglycerides and high concentrations of FFA can lead to insulin resistance in muscles and liver, weakened carbohydrate oxidation, reduced glucose storage in muscle and increased triglycerides, or hyperlipidemia [17], [18]. As most patients with T2DM are found to have a higher body fat percentage, a high fat diet (HFD) can cause β cells to produce insulin and lead to hyperinsulinemia and increased FFA [19]. Obesity and inactivity together are found to increase the predisposition of T2DM through the increase output of FFA from adipose tissue, an inflammatory mechanism, to decrease insulin sensitivity in insulin sensitive tissues and glucose tolerance [20]. The decrease in insulin sensitivity and glucose tolerance results in elevated blood glucose levels, insulin resistance, and adipokine dysregulation [21], [22].

Health Risks Associated with T2DM

Patients with T2DM have been found to be more susceptible to other diseases, such as cardiovascular disease, diabetic neuropathy, diabetic nephropathy and diabetic retinopathy [1], [23]. The specific cardiovascular disease associated with diabetes is diabetic cardiomyopathy, which results from change in myocardial structure and function caused by myocardial ischaemia, or reduced blood flow to heart muscles, and hypertension [23]. Out of all the associated diabetic diseases, cardiovascular disease affects approximately 32.3% of all patients with T2DM, making it the major cause of morbidity and mortality in diabetic patients [18], [23].

Sex Difference in Health Risks Associated with T2DM

Although patients with T2DM are more susceptible to other diseases, how T2DM affects a patient depends on their sex, as male and females are genetically, hormonally, and behaviorally different [24], [25]. According to the CDC's National Diabetes Statistics Report 2020, there are

more males with diabetes, but there are more females that are pre-diabetic [4]. An important factor to this sex difference is hormones. Sex hormones can impact an inflammatory response, body composition, metabolism, and vascular function [24]. As young females are protected by estradiol (E2) against HFD induced metabolic changes, the chance of developing T2DM and cardiovascular disease are reduced [24], [26]. However, once females undergo menopause, the protection from E2 is gone and diabetic females have a worsened cardiovascular disease outcome in comparison to diabetic males [19], [24], [25], [27]. Diabetic female mice also have an increase in regulatory T cells (Treg), which allows the for the maintenance of an anti-inflammatory environment in adipose tissue. Treg are specialized T cells that suppress immune response, preventing autoimmunity [28]. On the other hand, diabetic male mice develop inflammation in adipose tissue, glucose intolerance, hyperinsulinemia and hypertrophy [27].

Objective of the Study

With more information on the underlying mechanism behind the sex difference in T2DM still unknown, the goal of this project is to study the metabolic sex difference response to diet, a major environmental factor, and how it correlates to the development of T2DM. The project also aims to investigate the cause of the decreased oxidative phosphorylation found in T2DM. This experiment uses a HFD induced obesity model in mice to study T2DM development and progression by performing glucose tolerance tests (GTT) and echocardiograms, collecting serum, urine, and organs for metabolic analysis, respiratory analysis and other future experiments (**Table 1**). After 14 months of high-fat feeding, tissues will be investigated on a molecular level to see the respiratory reaction and mitochondrial morphology in the hippocampus, heart, pancreas, kidney, skeletal muscle, and liver.

MATERIALS AND METHODS

Ethical Statement

All animals were treated in compliance with *Guide for the Care and Use of Laboratory Animals* and experiments used protocols approved by the VA San Diego Healthcare System Institutional Animal Case and Use Committee (San Diego, CA).

Animals and Tissue Collection

8-week-old female and male wild-type C57BL/6 mice were purchased from Jackson Laboratory (Bar Harbor, ME). Animals were housed in a 12-hour light-dark cycle in a temperature-controlled environment with unlimited access to food and water. Then, the heart, hippocampus, kidney, liver, pancreas, and smooth muscle was collected. All terminal tissue collection took place on euthanized mice.

Treatment and Model

Animal assignment to specific experimental groups were blinded and randomized to treatment. Through an obesity driven T2DM model, 24 16-18 week old female and male mice were fed either a control LFD (10% kcal fat chow, D12451; Research diets, New Brunswick, NJ, USA) or a matched sucrose HFD (45% kcal fat chow, D12451; Research diets, New Brunswick, NJ, USA) (**Table 2**).

Body Weight and Food Intake

Body weight and food intake were monitored with food intake measured weekly and body weights weighed biweekly. The food intake was then calculated to find how much each mice ate daily (**Appendix A**).

3 Hour Fasting Blood Glucose and Serum Collection

Mice was fasted 3 hours prior to serum collection and blood glucose measurements. Mice were placed under isoflurane anesthesia (3-5% at a flow rate of 1 L/min oxygen) for 30-50 seconds before collecting blood for serum and measuring blood glucose via retro-orbital bleed.

Glucose Tolerance Tests (GTT),

Usually, two weeks after serum collections, glucose tolerance tests were performed on female and male mice fasted overnight for 10 hours after every 3 months of diet intake. Female and male were separated and GTT's were taken at separate times. Mice's body weight was measured before a lateral tail vein slice. Blood glucose levels were measured from the lateral tail vein using glucose test strips (Bayer Contour Diabetic Glucose Monitoring System with Blood Glucose Test Strips, Ascensia, Parsippany, NJ, USA). Mice were injected intraperitoneal (ip) with d-glucose (1 mg/g BW) an hour after initial lateral tail vein slice and measurement. Glucose levels were then measured through the lateral tail vein at 30, 60, 90, 120, and 180 minutes after glucose IP injection.

Ulcerative Dermatitis or Skin Lesion Treatment

Mice found with UD or skin lesions were recorded and treated with Hexa-Caine (2.46% lidocaine HCl, PRN Pharmacal, Inc., Pensacola, FL, USA) every day until no skin lesions were detected. Persistent cases of UD were treated by placing mice under isoflurane anesthesia (1-1.5% at a flow rate of 1 L/min oxygen) and trimming their nails.

High Resolution Mitochondrial Respirometry

Mitochondrial respiration was measured using the Oxygraph-2k (O2k, OROBOROS Instruments, Innsbruck, Austria). Up to seven O2K instruments (14 chambers) were used in parallel. Substrate-Uncoupler-Inhibitor-Titration (SUIT) protocol, specifically SUIT-014 O2 pfi D042, of oxidative phosphorylation (OXPHOS) substrates was used for mitochondrial activity. Specific sections of the kidney, liver and pancreas were taken from harvested organs and dissected so only 0.4-1.0mg of the tissue was place into the chamber. Only smooth muscle tissue was placed in 3-4mg saponin dissolved in BIOPS. The hippocampus was homogenized in BIOPS and injected into the chamber. Tissue is added to an Oxygraph-2k chamber with 2300uL of Miro5 buffer. O2 was injected and after the stabilization of the machine, a substrate is added in every 2 minutes. Substrates include malate and glutamate, adenosine diphosphate, cytochrome C, pyruvate, succinate, FCCP, rotenone, and Antimycin A. Glutamate, malate, and pyruvate were added to measure the LEAK respiration. Adenosine diphosphate was added to measure phosphorylating respiration and cytochrome C was added to measure the integrity of the mitochondria. To find electron transfer capacity, FCCP was added and complex I was eliminated through inhibition of back electron transfer using rotenone and triggered complex II activity by addition succinate. Then data was sorted and calculated (**Appendix B**).

Statistical Analysis

All data were analyzed using GraphPad Prism 9 software (GraphPad Software, Inc., San Diego, CA). Values are depicted as mean ± SEM and all cases $p<0.05$ was considered statistically significant. Statistical analysis was performed using a two-way ANOVA or three-way ANOVA followed by Tukey's multiple comparison test (two-tailed). Unless otherwise specified in the figure legend, *p < 0.05, **p < 0.01, ***p < 0.001, and ****p<0.0001 represent estimates of statistical significance.

RESULTS

T2DM Phenotype and Sex Difference

Body weight was measured from female mice on LFD, female mice on HFD, male mice on LFD and male mice on HFD biweekly for 74 weeks to monitor obesity development and to assess the effect of a high fat diet on weight gain (**Figure 1A**). Both male and female mice on HFD had an increase in body weight with males gaining weight rapidly and females more steadily. On the other hand, male and female mice on LFD remained steady at around 35-40g and 23-28g, respectively. Unexpectedly, female mice on HFD did not gain as much weight as the male mice on HFD. Males on HFD chow had a larger body weight increase and there was a significant difference ($p<0.05$) between them and every other group after 2 weeks of diet intake. On the other hand, the body weight increase in females on HFD was not as drastic and did not start showing significance against females on LFD until 6 weeks of diet intake ($p<0.05$). There was also a significant difference between the males and females on LFD and on HFD throughout the 74 week treatment ($p<0.05$).

Daily food intake was calculated from female mice on LFD, female mice on HFD, male mice on LFD and male mice on HFD from weekly measurement of food intake for 74 weeks (**Figure 1B**). Male mice ate more grams of food daily than females and mice on the HFD ate more than those on LFD. A two-way ANOVA was conducted that examined the effect of gender and diet on food intake. There was no statistically significant interaction between the effects of gender and diet on food intake ($F(1, 1776) = 0.08370$, $p= 0.7724$) but there was a statistically significant difference within gender, ($F(1, 1776) = 1013$, $p<0.0001$) and within diet, ($F(1, 1776) = 54.23$, $p<0.0001$). Post-hoc test revealed significant main effects among all groups ($p<0.0001$).

GTT's revealed that after 3 months of eating HFD, male and female mice on HFD started to exhibit altered glucose-tolerance response and hyperglycemia, when compared to their respective controls (**Figure 2A**). A three-way ANOVA was conducted, but there was no statistically significant three-way interaction between time, gender and diet ($F(5, 460) = 1.299$, $P=0.2631$). However, there was statistical significance between time and gender ($F(5, 460) = 4.738$, $P=0.0003$), time and diet ($F(5, 460) = 14.53$, $P<0.0001$), and diet and chow ($F(1, 92) = 12.24$, $P=0.0007$). After 6 months of treatment, males and females on HFD maintained their altered glucose-tolerance response, but males and females on LFD also had a slight increase in glucose intolerance (**Figure 2C**). A three-way ANOVA found statistical significance between time, gender, and diet ($F(5,460) = 8.611$, $P<0.0001$). At 9 months treatment, male and females on HFD had extremely similar altered glucose-tolerance responses, where it took a longer time for glucose levels to return back to baseline (**Figure 2E**). However, there was a decrease in the peak amount of blood glucose found in male and females on HFD, leading only to having significance between chow and time ($F(5, 435) = 3.918$, $P=0.0017$). At 12 months of treatment, male and female on HFD maintained their closely similar altered glucose-tolerance response and decrease in amount of blood glucose after ip injection (**Figure 2G**). A 3-way ANOVA only reveled a significant difference between gender and time ($F(5, 430) = 3.088$, $P=0.0095$). At the time of 3 and 6 month GTT, the body weight of males were larger than those of the females ($p<0.05$) and the weight of the mice on HFD were greater than those on LFD ($p<0.0001$) (**Figure 2B and 2D**). At 6 months GTT, females on HFD had a larger body weight than males on LFD, but there was only a significant difference at the 12 month GTT ($p<0.01$) (**Figure 2F and 2H**). The weight difference between a HFD and LFD remained statistically significant ($p<0.0001$).

Ulcerative Dermatitis Development

Within 7 weeks of treatment, the first case of UD was found in females on HFD. Of the 96 mice treated, 29 mice developed UD or wounds from fighting (**Figure 3A**). Majority of the mice that developed UD were females on HFD, when compared to females on LFD ($P < 0.001$), males on LFD ($P < 0.001$), and males on HFD ($P < 0.0001$). In the end there were a total of 45 cases of UD and skin lesions, with 16 of those cases being recurring (**Figure 3C and 3D**). Of the 45 cases, 15 of these cases were either from females on HFD or males on LFD. There was only a significant difference between male's on HFD and the other three groups ($p<0.01$). Then, within those 45 cases male's on LFD had 8 recurring cases, while female's on LFD had 6 cases of recurring cases. One way to treat the UD and skin lesions was to trim the mice's nails (**Figure 3B**). The female nails had to be trimmed more than the male's, but the mice on LFD had to have their nails trimmed more than those on HFD ($p<0.05$).

Effects of Mitochondrial Function

A high-resolution oxygraphy was used to calculate mitochondrial respiration through oxygen flux on the heart, hippocampus, kidney, liver, pancreas, and smooth muscle (**Figure 4**). *Post hoc* test revealed only the heart had a difference in mitochondrial respiratory capacity between males and female on HFD, females on LFD and males on LFD, and females on HFD and males on LFD (**Figure 4A**). Mitochondrial respiration through complex I (CI) and complex II (CII) oxidative phosphorylation (OXPHOS) and CI and CII electron transfer capacity was higher in female hearts. On the other hand, there was no significant difference found in the hippocampus, kidney, liver, pancreas, and smooth muscle. However, a three-way interaction between mitochondrial respiration, chow and gender ($F(6, 114) = 2.237$, $P=0.0445$) and a two-way interaction between mitochondrial respiration and gender ($F(6, 114) = 2.396$, $P=0.0323$) was

found in the hippocampus (**Figure 4B**). In the kidney, a three-way interaction between mitochondrial respiration, chow and gender was found to be statistically significant ($F(6, 66) = 2.464$, $P=0.0328$) (**Figure 4C**). The liver and pancreas revealed a statistically significant interaction between mitochondrial respiration and gender ($F(6, 126) = 2.371$, $P=0.0333$, $F(6, 60) = 4.556$, $P=0.0007$) (**Figure 4D and 4E**). However, there was no significant interaction in the smooth muscle (**Figure 4F**).

Results is coauthored with Zemljic-Harpf, Alice E.; Walker, Emily; Nazarbegian, Melody; Devulapalli, Swetha; Fannon, McKenzie J; Lu, Sun; Roth, David M.; Patel, Hemal H. The thesis author was the primary author of this paper.

DISCUSSION

Weight was monitored over the course of 74 weeks to assess the effect of a high fat diet on weight gain. Overall, males on HFD showed a 79% increase and females on HFD showed a 91% increase in body weight. Like previous studies, male's on HFD had the fastest weight gain and was overweight at a younger age, while females on HFD did not gain as much weight rapidly, but had a greater increase in body weight as they got older [30]. The difference in weight gain between the males and females, especially when females on HFD did not gain a significant amount of weight until l6 weeks of diet intake, is because young female mice are protected by estrogen from high-fat induced metabolic syndrome [26], [27]. Louet and colleagues have shown that when female mice are postmenopausal, they start to develop obesity and insulin resistance, and therefore are at a higher risk for T2DM [26]. Female mice reach perimenopause, or the transitional state into menopause, when they are 9 months old and are reproductively senescent, or a state of reproductive decline, from 9 to12 months of age [29]. Therefore, the female mice on HFD, who were 16-18 weeks old, when treatment began, were considered to be reproductively senescent or postmenopausal at 18-30 weeks treatment time, explaining the steeper slop of weight gain and ultimately the 91% increase in body weight at the end of the study. However, without being able to conduct tests to confirm whether the female mice were reproductively senescent or postmenopausal, we cannot completely attribute the decreased weight gain in young female mice to the protection by estradiol (E2). E2 has been found to prevent lipid accumulation in the liver, promote fatty acid oxidation and the survival of β cells in conditions that promote oxidative injuries [26]. Therefore, by running an E2 test on serum from different time periods, we can correlate body weight and the amount of estrogen to confirm that E2 is one of the underlying reason why females on HFD did not have a significant difference from females on

LFD until they were reproductively senescent. An E2 test run on serum collected at 6 months of treatment should reveal a decreased level of E2 in the middle-aged female mice, when comparing to the serum taken at baseline, indicating the mice are postmenopausal. Consequently, an E2 test should also indicate low levels of E2 in male mice at all time points, indicating the males are missing the E2 protecting and being one of the reasons why there is a significant difference in the impact of a high fat diet between male mice on HFD and male mice on LFD.

Another contributing factor to the difference in weight gain between males and females was the amount of food eaten per day, as males ate significantly more. Since males on HFD are eating more fat and having a higher energy intake from their diet compared to a LFD, they are going to gain a higher excess body weight than females. Besides hormone protection and the amount of food eaten, there may also be underlying metabolic factors can be an influencing factor on why there is a significant difference on the effect of high fat for males on different diets and also between sexes. Therefore, a lipid and liver tox panel should also be performed on the serum collected to determine any differences in triglycerides, cholesterol, low density lipoprotein (LDL) cholesterol, high density lipoprotein (HDL) cholesterol, aspartate aminotransferase (AST), alanine transaminase (ALT), alkaline phosphatase (ALP), gamma-glutamyl transferase (GGT), bile acids and total bilirubin. From the lipid panel, both male and female mice on HFD should display increased levels of triglycerides and LDL cholesterol, but lower levels of HDL cholesterol, which are associated with the insulin resistance syndrome in T2DM [31]. LDL cholesterol is known to build up on the walls of blood vessels, narrowing blood flow, and triglycerides, which is a type of lipid converted from excess calories, in high levels explains the atherosclerosis found in T2DM patients [32]. On the other hand, HDL cholesterol has been found to be anti-inflammatory and pro-vasodilatory, so having high levels of HDL has been found to

have an inverse relationship with the risk of cardiovascular disease [32]. Therefore, male, and female mice on LFD should not display high levels of LDL cholesterol and triglycerides, in comparison to those on HFD. On the other hand, a liver tox panel can show the effect of T2DM has on the liver, as the liver plays an important role in glycogenolysis and gluconeogenesis. The mice on HFD should display abnormalities in their liver tox panels when compared to the mice on LFD, as lipid accumulation in hepatocytes indicates insulin resistance [33], [34]. If the mice on HFD had developed nonalcoholic fatty liver disease (NAFLD), they should display higher levels of ALT, AST and GGT, which are all enzymes that are released when the liver is damaged [33].

The onset of T2DM is distinguished by insulin resistance and β-cell compensation, which ultimately leads to β-cell exhaustion and hyperglycemia [35]. Although an insulin tolerance test was not performed, a GTT was performed to check for hyperglycemia and altered glucose tolerance. High fasting blood glucose levels after 3 months of diet ingestion are consistent with the onset of T2DM. Male mice on HFD also started to display altered glucose-tolerance response, while females appeared to have an altered glucose-tolerance, but due to the female mice being unfamiliar with human interaction it caused their altered blood glucose. However, starting at 9 months of diet ingestion, both female and male mice on HFD displayed altered glucose tolerance and hyperglycemia, but did not exhibit high levels of blood glucose after ip injection of glucose. It is suspected that both males and females started to urinate glucose or have glucosuria. Glucosuria occurs when the blood glucose concentration reaches the renal threshold [36]. Renal threshold can be affected by a multitude of factors, such as hyperglycemia, age, and heart failure [36]. In a normally functioning kidney, the renal tubule will reabsorb the glucose present in the glomerular filtrate, but when someone has T2DM, the glomerular filtrate rate is

reduced and therefore delivery of glucose to the tubule is reduced when the high blood glucose surpasses the capacity of the renal tubule to reabsorb it [37]. Therefore, to determine whether glucosuria is occurring, urine samples taken at 9 months of treatment should be test. Overall, males on HFD continuously displayed an altered glucose tolerance, while females were originally protected, but ultimately caught up to the males on HFD.

Another recurring issue with the high fat diet was the cases of UD and skin lesions. It is known in C57BL/6 are more prone to UD, especially as they age [38], [39]. UD is the inflammation of the skin and is an inflammation phenotype that can be caused by open wounds from fighting and excessive sheering and scratching [38]. Obesity-inducing diets have previously found to increase the incidence of UD, which is why there was a higher incidence of females on HFD to developed UD, as obesity-inducing diets have been found to increase the incidence of UD [40]. As females had a higher number of cases, it could be reasoned that females are more sensitive to the HFD than males are. However, males on LFD did have a high number of cases of UD, but unlike the females, who are thought to have a higher incident rate due to sensitivity to the diet, males on LFD were mostly caused by fight wounds. This can be related to biobehavioral as males on HFD were not as active and appeared to have a more mellow personality, while males on LFD were active and fighting. To check the affect UD had on the mice, a serum cytokine analysis can be performed to test for TNFα, IL6, IL1α, keratinocyte-derived cytokine (KC), macrophage chemotactic protein 1 (MCP1), normal T-cell expressed and secreted (RANTES) protein [41]. Like previous studies, female mice on HFD with UD had should show increased levels of acute inflammatory cytokines IL6, TNFα, and KC, but lower levels of leukocyte chemoattractant RANTES [41]. To treat mice with UD, mice were sprayed with Hexa-Caine every day until the skin lesions disappeared. However, it took a longer amount of time for

the mice to heal as Hexa-Caine is a topical anesthetic spray that only desensitizes the area. Therefore, in addition to spraying with Hexa-Caine, the mice's nails were trimmed, as it has been found to heal UD faster and prevent the mice from developing UD as quickly [42],[43]. The combination of spraying and nail trims allowed to not only recover within 2 days, but also prevented the development of UD for about a month.

Based on the GTT, it appeared the mice on HFD had hyperglycemia. Hyperglycemia had been found to be directly related to oxidative stress, where high levels of glucose causes the mitochondria to produce more ROS and prompt oxidative stress and tissue damage [44], [45]. However, when looking at the mitochondrial respiration in the hippocampus, kidney, liver, pancreas, and smooth muscle, there was no defects found in mitochondrial respiratory capacity between mice on HFD and LFD. This was unexpected as previously studies have found a decrease in mitochondrial respiration in skeletal muscle, increased oxidation in fatty livers with nonalcoholic steatohepatitis, which is commonly found with T2DM, and increased ROS in pancreatic β-cells [44], [46], [47], [48], . There was, however, a sex difference between mice on HFD in mitochondria found in the heart. Females appeared to have a higher respiration in electron transfer capacity than those of males. Despite the difference between sexes, there was no significant difference between males on LFD and HFD and females on LFD and HFD. This is similar to a previous study, which found unchanged oxidative phosphorylation [49]. However, the effect on mitochondrial between sexes can further be examined by examining mitochondrial density and examining the number of mitochondria in each tissue through transmission electron microscopy (TEM).

CONCLUSION

This thesis presented preliminary findings in the metabolic sex difference response to diet. Females displayed a delayed response to a HFD, but eventually developed T2DM. Based on their weight progression, both males and females on HFD had more than a 70% increase in weight, where males rapidly gained weight, but the females steadily gained weight. By the end of the treatment, mice on HFD displayed hyperglycemia and altered glucose tolerance. Female mice, especially those on HFD, developed dermatitis due to their sensitivity to high fat diets. However, males on LFD also developed dermatitis from fight wounds, as they were more active than the relaxed males on HFD. Further findings in the metabolic sex difference response to diet can be found by analyzing echocardiograms, as cardiovascular disease is associated with T2DM. The echocardiograms could reveal larger hearts, especially in the left ventricle, indicating an increased risk of heart failure in mice on high fat diet. Serum can also be analyzed through a metabolic panel, hormone panel and inflammation panel to reveal further connections. Urine analysis can also reveal if there is protein in the urine, as it is associated with T2DM.

This thesis also presented preliminary findings in the sex difference in mitochondrial respiration in the heart. In the heart, there was no significant difference between diets, but only a sex difference in CI and CII OXPHOS and electron transfer capacity between males and females, where females had a higher response than males. Further viewing of the mitochondria through transmission electron microscopy would show if there are any morphological differences. In addition, RNA-sequencing on the hearts can reveal which genes are turned on or if there is a sex difference in expression level.

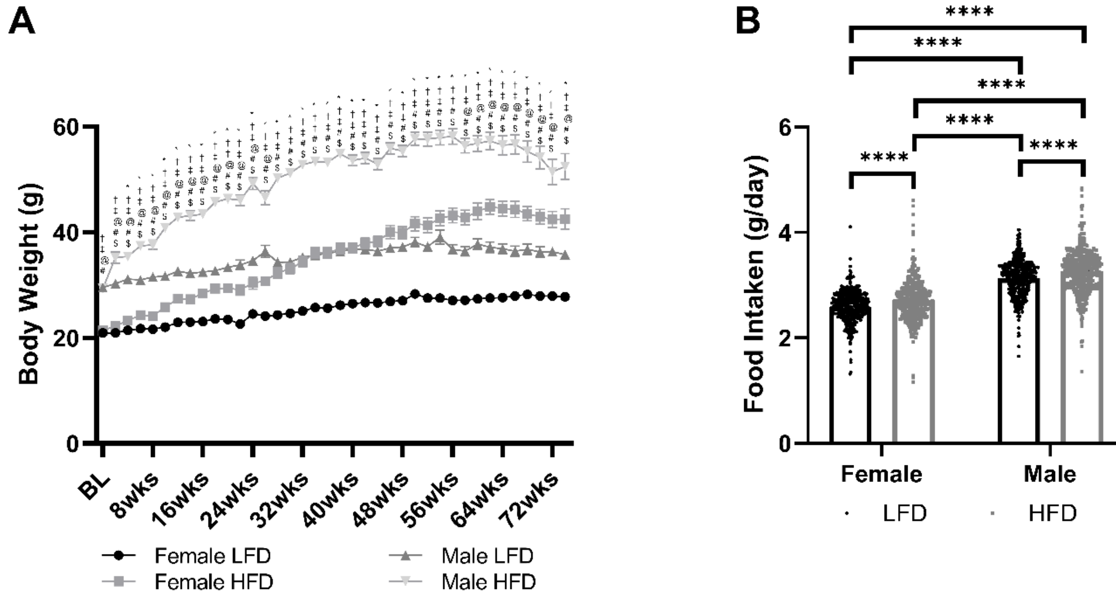
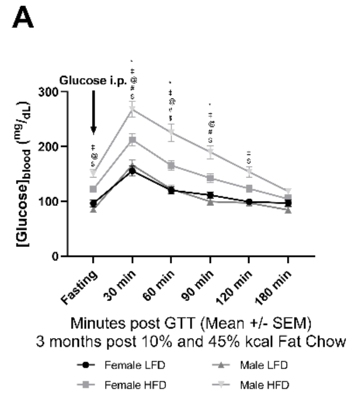


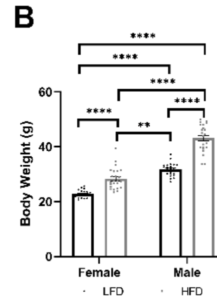
Figure 1. Body weight and food intake in female and male on low-fat diet (LFD) or high-fat diet (HFD). (**A**) Body weight in LFD female (n≥23), HFD female (n≥23), LFD male (n≥22), and HFD male (n≥22) measured at different time points over 74 weeks of treatment. (**B**) Average food intake in a day by a LFD female (n≥23), HFD female (n≥23), LFD male (n≥22), and HFD male (n≥22) over 74 weeks. P<0.05 for Female LFD compared with Female HFD (*), Female LFD compared with Male LFD (†), Female LFD compared with Male HFD (‡), Female HFD compared with Male LFD (@), Female HFD compared with Male HFD (#), and Male LFD compared with Male HFD ($).

Figure 2. Effects of a high-fat diet (HFD) during Glucose Tolerance Test (GTT). (**A**) GTT in fasted LFD female (n=24), HFD female (n=24), LFD male (n=24), and HFD male (n=24) after 3 months of LFD or HFD. (**B**) Body weight during GTT of LFD female (n=24), HFD female (n=24), LFD male (n=24), and HFD male (n=24) after 3 months of LFD or HFD. (**C**) GTT in fasted LFD female (n=24), HFD female (n=24), LFD male (n=24), and HFD male (n=24) after 6 months of LFD or HFD. (**D**) Body weight during GTT of LFD female (n=24), HFD female (n=24), LFD male (n=24), and HFD male (n=24) after 6 months of LFD or HFD. (**E**) GTT in fasted LFD female (n=23), HFD female (n=23), LFD male (n=23), and HFD male (n=22) after 9 months of LFD or HFD. (**F**) Body weight during GTT of LFD female (n=23), HFD female (n=23), LFD male (n=23), and HFD male (n=22) after 9 months of LFD or HFD. (**G**) GTT in fasted LFD female (n=23), HFD female (n=23), LFD male (n=22), and HFD male (n=22) after 12 months of LFD or HFD. (**H**) Body weight during GTT of LFD female (n=23), HFD female (n=23), LFD male (n=22), and HFD male (n=22) after 12 months of LFD or HFD. $P<0.05$ for Female LFD compared with Female HFD (*), Female LFD compared with Male LFD (†), Female LFD compared with Male HFD (‡), Female HFD compared with Male LFD (@), Female HFD compared with Male HFD (#), and Male LFD compared with Male HFD ($).

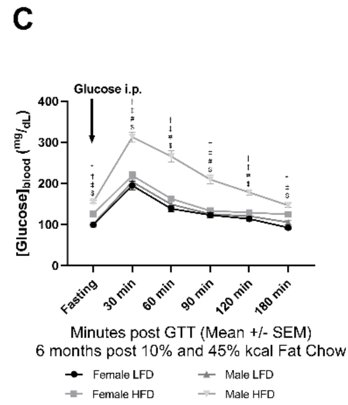
A



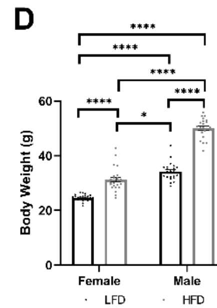
B



C



D



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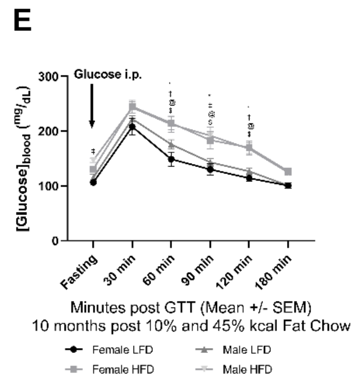
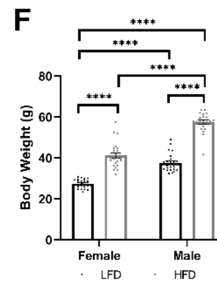
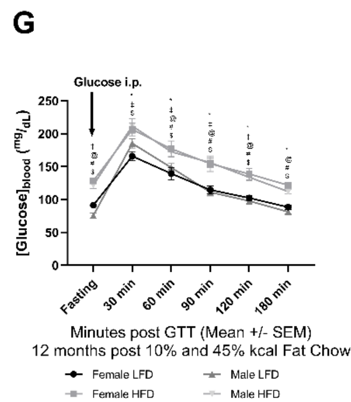


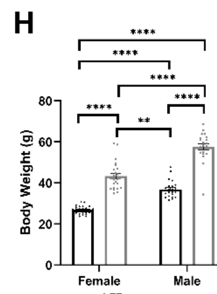
F



G



H



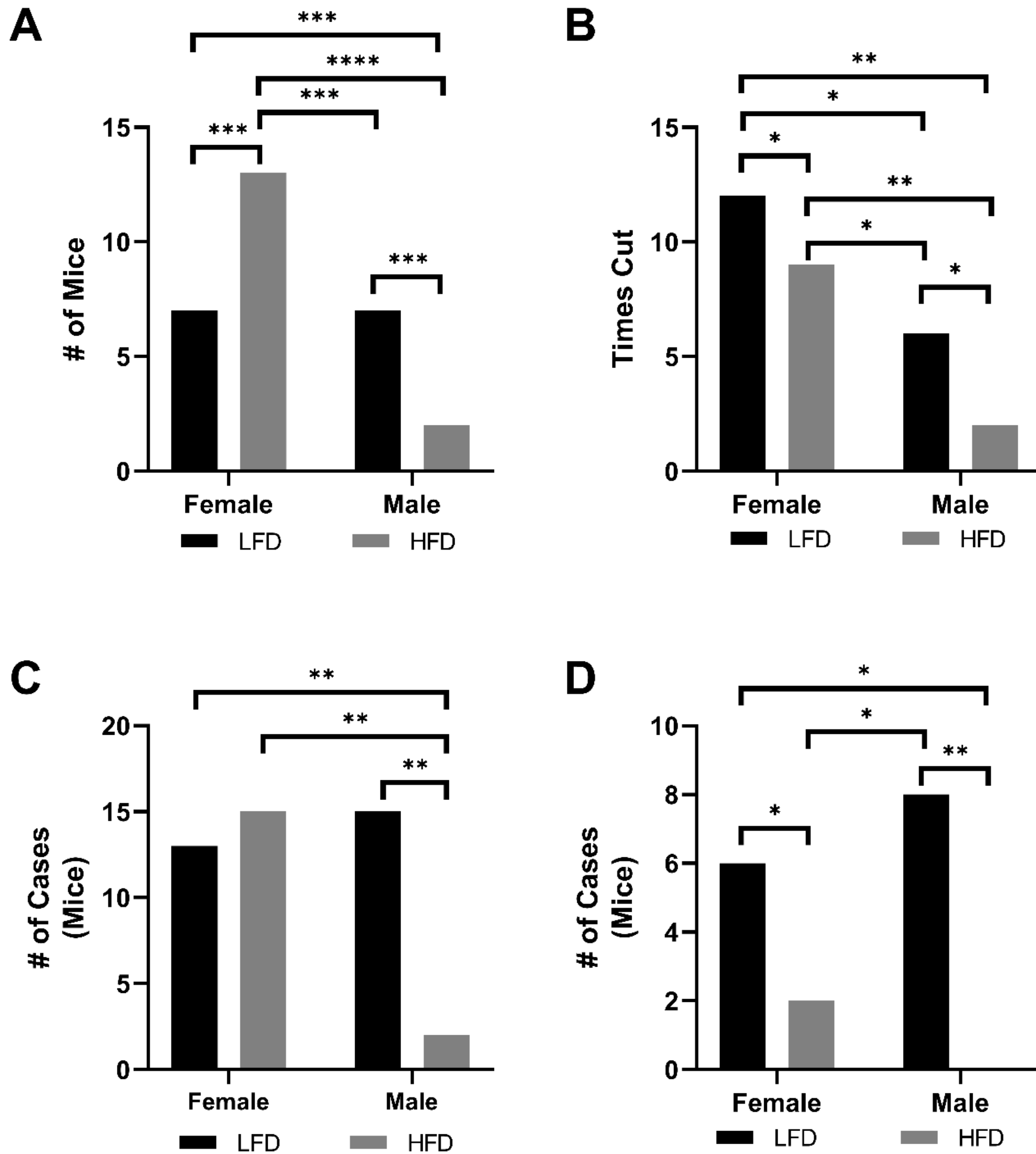


Figure 3. Effects of high-fat diet (HF) on development of ulcerative dermatitis or skin lesion. (**A**) Number of mice that developed dermatitis or skin lesions in LFD female (n=24), HFD female (n=24), LFD male (n=24), and HFD male (n=24). (**B**) Number of times mice nails were trimmed as treatment to dermatitis or skin lesions in LFD female (n=24), HFD female (n=24), LFD male (n=24), and HFD male (n=24). (**C**) Total cases of dermatitis or skin lesions in LFD female (n=24), HFD female (n=24), LFD male (n=24), and HFD male (n=24). (**D**) Number of recurring cases of dermatitis or skin lesions in LFD female (n=24), HFD female (n=24), LFD male (n=24), and HFD male (n=24).

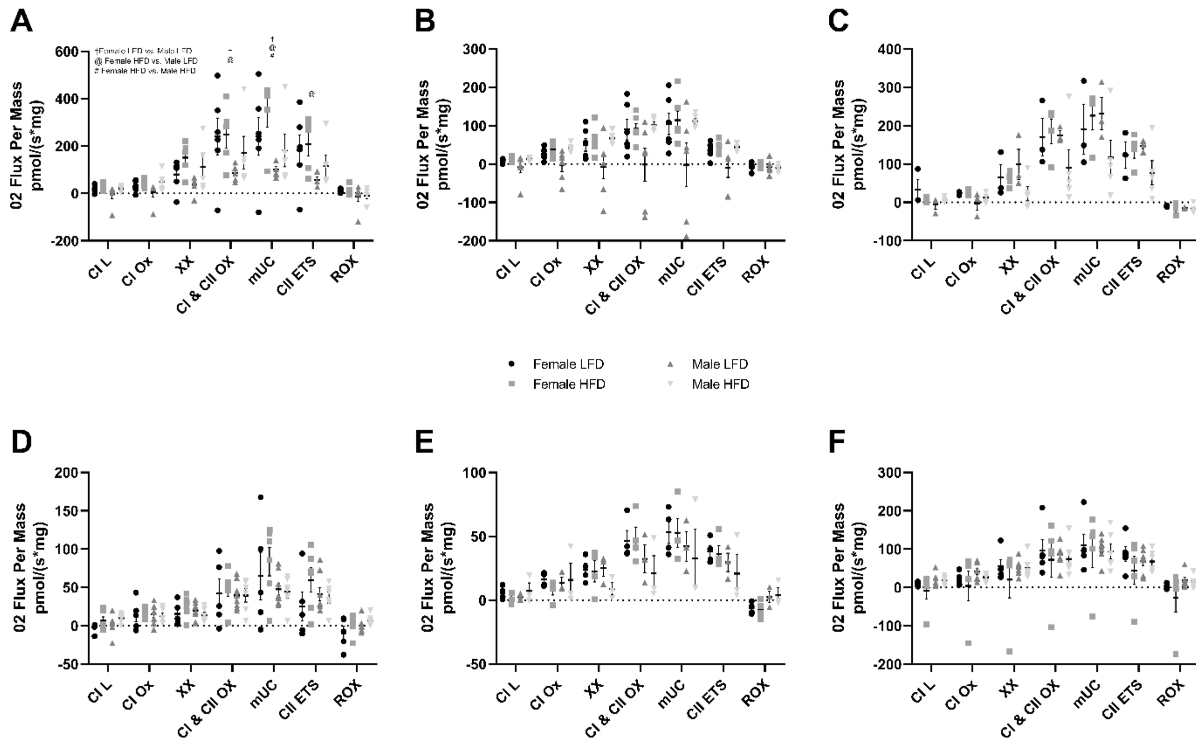


Figure 4. Effects of high-fat diet (HFD) on mitochondrial respiration in various organs. Respiratory flux measured in (**A**) heart, (**B**) hippocampus, (**C**) kidney, (**D**) liver, (**E**) pancreas, and (**F**) smooth muscle in LFD female (n≥3), HFD female (n≥4), LFD male (n≥ 3), and HFD male (n≥ 3) after 74 weeks of treatment.. Oxygen flux expressed as mass-specific respiratory capacity (pmol·s-1·mg-1) for leak respiration (CI L), complex I respiration (CI Ox), complex I and II respiration combined (CI & CII OX), electron transfer capacity (mUC), electron transfer capacity of complex II (CII ETS), and residual oxygen consumption (ROX). Data are means ± SEM.

TABLES

Table 1. Experimental Design and Collection Date

Time Point	GTT	Serum Collection	Urine	Echo	Oroboros	Organ Collection
0 Months		X				
3 Months	X					
6 Months	X	X				
9 Months	X		X			
12 Months	X	X		X		
17 Months		X			X	X

Table 2. Nutritional Makeup of each Chow Diet

Treatment	Protein (kcal%)	Fat (kcal%)	Carbohydrate (kcal%)	Energy Density (Kcal/g)
Control				
10% Chow (n=48)	20	10	70	3.82
T2DM				
45% Chow (n=48%)	20	45	35	4.7

APPENDIX

Appendix A. foodSorter.py Source Code. Code used to calculate daily food intake and sort into different groups.

```
1.  # importing openpyxl module to read excel file and xlswriter to write new excel file
2.  import openpyxl as xl;
3.  import xlsxwriter
4.
5.
6.  # getting file names
7.  inputfile = './Copy of 2nd T2DM Sex Comparison Study Intake & BW.xlsx'
8.  outputfile = './Sorted Food Intake.xlsx'
9.
10. #openeing the file we are reading
11. readingFile = xl.load_workbook(inputfile)
12. blueFW = readingFile.worksheets[2]
13. greenFW = readingFile.worksheets[3]
14.
15. # opening the file we are creating
16. #finishedSheet = xl.load_workbook(outputfile)
17. #compliedSheet = finishedSheet.active
18. workbook = xlsxwriter.Workbook(outputfile)
19. compliedSheet = workbook.add_worksheet()
20.
21. # Getting total number of rows and columns for Blue Room and Green Room Sheet
22. maxRowBlue = blueFW.max_row
23. maxColumnBlue = blueFW.max_column
24. maxRowGreen = greenFW.max_row
25. maxColumGreen = greenFW.max_column
26.
27.
28. #Writing Headers
29. compliedSheet.write(0, 0, 'Female')
30. compliedSheet.write(0, 1, 'Female')
31. compliedSheet.write(0, 2, 'Male')
32. compliedSheet.write(0, 3, 'Male')
33. compliedSheet.write(1, 0, '10%')
34. compliedSheet.write(1, 1, '45%')
35. compliedSheet.write(1, 2, '10%')
36. compliedSheet.write(1, 3, '45%')
37. compliedSheet.write(2, 0, 'Total g Eaten')
38. compliedSheet.write(2, 1, 'Total g Eaten')
39. compliedSheet.write(2, 2, 'Total g Eaten')
40. compliedSheet.write(2, 3, 'Total g Eaten')
41.
42. #Create lists
43. female10 = []
44. female45 = []
45. male10 = []
46. male45 = []
47.
48.
49.
50. #Loop through all cells in the Blue sheet.
51. for i in range(6, maxRowBlue+1):
52.     for j in range(1, maxColumnBlue+1):
53.         currCell = str(blueFW.cell(row = i, column = j).value)
54.         #Checks that the cell is the Cage Number
55.         if 'Cage ' in currCell and 'per 1 days' not in currCell:
```

```
56.             cageStr = currCell
57.             cageStr = cageStr[4:]
58.             # Getting Cage number into int
59.             cageNum = int(cageStr)
60.             # Getting the percentage chow (treatment)
61.             chowPer = str(blueFW.cell(row = i, column = j+1).value)
62.             #Checks to make sure there is a value for the New food amount and Old food
    amount
63.             if blueFW.cell(row = i, column = j+2).value and blueFW.cell(row = i, column
     = j+3).value:
64.                 #Convert New food amount and Old food amount to be a float
65.                 newFood = float(blueFW.cell(row = i, column = j+2).value)
66.                 oldFood = float(blueFW.cell(row = i, column = j+3).value)
67.                 #Checks that there is more New Food than Old Food so there is no negati
    ve value
68.                 if(oldFood < newFood):
69.                     #Gets amount of food each cage ate in a week
70.                     weeklyFood = newFood - oldFood
71.                     #Gets the number of days in that week
72.                     numDayStr = blueFW.cell(row = i, column = j+5).value
73.                     numDay = float(numDayStr[-1])
74.                     #Gets number of mice in the cage
75.                     numMouseStr = blueFW.cell(row = i, column = j+6).value
76.                     numMouse = float(numMouseStr[-1])
77.                     #Calculates how much food each cage ate each day
78.                     dayFood = weeklyFood/numDay
79.                     #Calculates how much food each mice ate per day
80.                     foodValue = dayFood/numMouse
81.
82.                     #Checks to see if the cage is female or male
83.                     if cageNum <= 18:
84.                         #Checks the diet type to add to the approiate list
85.                         if chowPer == "0.1":
86.                             female10.append(foodValue)
87.                         else:
88.                             female45.append(foodValue)
89.                     else:
90.                         #Checks the diet type to add to the approiate list
91.                         if chowPer == "0.1":
92.                             male10.append(foodValue)
93.                         else:
94.                             male45.append(foodValue)
95.                 else:
96.                     continue
97.             else:
98.                 continue
99.         else:
100.                continue
101.
102.
103.    #Loop through all cells in the Green sheet. Same as above but with a different s
    heet.
104.    for i in range(6, maxRowGreen+1):
105.        for j in range(1, maxColumGreen+1):
106.            currCell = str(greenFW.cell(row = i, column = j).value)
107.            if 'Cage ' in currCell and 'per 1 days' not in currCell and 'pass away'
    not in currCell:
108.                cageStr = currCell
109.                cageStr = cageStr[4:]
110.                cageNum = int(cageStr)
111.                chowPer = str(greenFW.cell(row = i, column = j+1).value)
```

```
112.
113.                    if greenFW.cell(row = i, column = j+2).value and greenFW.cell(row =
    i, column = j+3).value:
114.                        newFood = float(greenFW.cell(row = i, column = j+2).value)
115.                        oldFood = float(greenFW.cell(row = i, column = j+3).value)
116.                        if(oldFood < newFood):
117.                            weeklyFood = newFood - oldFood
118.                            numDayStr = greenFW.cell(row = i, column = j+5).value
119.                            numDay = float(numDayStr[-1])
120.                            numMouseStr = greenFW.cell(row = i, column = j+6).value
121.                            numMouse = float(numMouseStr[-1])
122.                            dayFood = weeklyFood/numDay
123.                            foodValue = dayFood/numMouse
124.
125.                    if cageNum <= 6:
126.                        if chowPer == '0.1':
127.                            female10.append(foodValue)
128.                        else:
129.                            female45.append(foodValue)
130.                    else:
131.                        if chowPer == "0.1":
132.                            male10.append(foodValue)
133.                        else:
134.                            male45.append(foodValue)
135.                else:
136.                    continue
137.
138.        #Starts filling out the output Excel sheet with wanted data. Starts with Females
     at 10% diet.
139.        outputRow = 3
140.        outPutColumn = 0
141.        totalValue = 0
142.        num = 0
143.        #Loops through each value in the list and places it on the excel sheet
144.        for value in female10:
145.            compliedSheet.write(outputRow, outPutColumn, value)
146.            #Getting total of all values to calculate average
147.            totalValue = totalValue + value
148.            outputRow = outputRow + 1
149.            #Geting the number of values in the list to calculate average
150.            num = num + 1
151.        #Calculate Average to place into excel sheet.
152.        average = totalValue/num
153.        compliedSheet.write(outputRow+4, outPutColumn, 'average')
154.        compliedSheet.write(outputRow+5, outPutColumn, average)
155.
156.        outputRow = 3
157.        outPutColumn = 1
158.        totalValue = 0
159.        num = 0
160.        for value in female45:
161.            compliedSheet.write(outputRow, outPutColumn, value)
162.            outputRow = outputRow + 1
163.            totalValue = totalValue + value
164.            num = num + 1
165.        average = totalValue/num
166.        print(average)
167.        compliedSheet.write(outputRow+4, outPutColumn, 'average')
168.        compliedSheet.write(outputRow+5, outPutColumn, average)
169.
170.        outputRow = 3
```

```
171.        outPutColumn = 2
172.        totalValue = 0
173.        num = 0
174.        for value in male10:
175.            compliedSheet.write(outputRow, outPutColumn, value)
176.            outputRow = outputRow + 1
177.            totalValue = totalValue + value
178.            num = num + 1
179.        average = totalValue/num
180.        compliedSheet.write(outputRow+4, outPutColumn, 'average')
181.        compliedSheet.write(outputRow+5, outPutColumn, average)
182.
183.        outputRow = 3
184.        outPutColumn = 3
185.        totalValue = 0
186.        num = 0
187.        for value in male45:
188.            compliedSheet.write(outputRow, outPutColumn, value)
189.            outputRow = outputRow + 1
190.            totalValue = totalValue + value
191.            num = num + 1
192.        average = totalValue/num
193.        compliedSheet.write(outputRow+4, outPutColumn, 'average')
194.        compliedSheet.write(outputRow+5, outPutColumn, average)
195.
196.        workbook.close()
```

Appendix B. oroborosProgram.py Source Code. Code used to sort Oroboros data from machine and calculate each data point to be graphed.

```
1.  # importing openpyxl module to read excel file and sys to allow for user input
2.  import openpyxl as xl;
3.  import sys, re
4.
5.
6.  # getting file names
7.  inputfile = sys.argv[1]
8.
9.  #openeing the file we are reading
10. excelFile = xl.load_workbook(inputfile)
11.
12. #Create Dictionary to store Lists of data
13. masterDict = {}
14. masterDict["Animal - Organ"] = []
15. masterDict["Machine"] = []
16. masterDict["Unit"] = []
17.
18.
19. #Setting up Variables to be used in for loop. mostRow to remember which row
20. #had the most number of Columns. maxNumCol to remember the max number of columns
21. #mostColumnWS to remember sheet containins the most number of columns
22. mostColumnWS = excelFile.worksheets[1]
23. mostRow = 1
24. maxNumCol = 0
25.
26. #Searching the sheet for the sheet and row with most columns to set up Dict
27. for sheet in excelFile.sheetnames:
28.     currSheet = excelFile[sheet]
29.     maxRow = currSheet.max_row
30.     maxColumn = currSheet.max_column
```

```
31.     for i in range(1, maxRow+1):
32.             currNumCol = 0
33.             if currSheet.cell(row = i, column = 1).value and currSheet.cell(row = i, column = 2).value is None:
34.                 for j in range(4, maxColumn+1):
35.                     if currSheet.cell(row = i, column = j).value:
36.                         currNumCol = currNumCol + 1
37.                     else:
38.                         continue
39.                 if currNumCol > maxNumCol:
40.                     maxNumCol = currNumCol
41.                     mostRow = i
42.                     mostColumnWS = currSheet
43.             else:
44.                 continue
45.
46. #Setting up all the Lists to place into the Dict
47. for cell in range(4, maxNumCol+4):
48.     name = mostColumnWS.cell(row = mostRow, column = cell).value
49.     masterDict[name.upper()] = []
50.
51.
52. headerRow = 1
53. tempDict = {}
54. for sheet in excelFile.sheetnames:
55.     if sheet != "Summary":
56.         currSheet = excelFile[sheet]
57.         #getting max number of columns and rows
58.         maxColumn = currSheet.max_column
59.         maxRow = currSheet.max_row
60.         for i in range(1, maxRow+1):
61.             tempDict = {}
62.             if currSheet.cell(row = i, column = 1).value and currSheet.cell(row = i, column = 4).value is None and currSheet.cell(row = i+1, column = 2).value is None and currSheet.cell(row = i+3, column = 2).value:
63.                 currList = masterDict.get("Animal - Organ")
64.                 currList.append(currSheet.cell(row = i, column = 1).value)
65.             elif currSheet.cell(row = i, column = 1).value == "X":
66.                 currList = masterDict.get("Machine")
67.                 currList.append(currSheet.cell(row = i, column = 2).value)
68.                 currList = masterDict.get("Unit")
69.                 currList.append(currSheet.cell(row = i, column = 3).value)
70.                 for j in range(4,maxColumn+4):
71.                     if currSheet.cell(row = headerRow, column = j).value:
72.                         headerName = currSheet.cell(row = headerRow, column = j).value

73.                         dataValue = currSheet.cell(row = i, column = j).value
74.                         tempDict[headerName.upper()] = dataValue
75.                 for key in masterDict:
76.                     if key != "Animal - Organ" and key != "Machine" and key != "Unit" and key in tempDict:
77.                         currList = masterDict.get(key)
78.                         currList.append(tempDict.get(key))
79.                     elif key !=  "Animal - Organ" and key !=  "Machine" and key != "Unit" and key not in tempDict:
80.                         currList = masterDict.get(key)
81.                         currList.append(None)
82.                     else:
83.                         continue
84.             elif currSheet.cell(row = i, column = 1).value and currSheet.cell(row = i, column = 4).value:
```

```
85.                 headerRow = i
86.             else:
87.                 continue
88.         else:
89.             continue
90.
91. print(masterDict)
92.
93. colNum = 1
94. if 'Summary' in excelFile.sheetnames:
95.     excelFile.remove(excelFile['Summary'])
96. excelFile.create_sheet('Summary')
97. currSheet = excelFile['Summary']
98. for key in masterDict:
99.     rowNum = 1
100.            currSheet.cell(row = rowNum, column = colNum).value = key
101.            currList = masterDict.get(key)
102.            for value in currList:
103.                rowNum = rowNum + 1
104.                currSheet.cell(row = rowNum, column = colNum).value = value
105.            colNum = colNum + 1
106.        excelFile.save(inputfile)
```

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