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A DIET ENRICHED IN STEARIC ACID PROTECTS AGAINST THE PROGRESSION OF TYPE 2 DIABETES IN LEPTIN RECEPTOR DEFICIENT MICE (DB/DB)

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A DIET ENRICHED IN STEARIC ACID PROTECTS AGAINST THE PROGRESSION OF TYPE 2 DIABETES IN LEPTIN RECEPTOR DEFICIENT MICE (DB/DB)

DISSERTATION

A dissertation submitted in partial fulfillment of the
requirements for the degree of Doctor of Philosophy in the
College of Medicine, Department of Physiology at the
University of Kentucky

By
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2012

ABSTRACT OF DISSERTATION

A DIET ENRICHED IN STEARIC ACID PROTECTS AGAINST THE PROGRESSION OF TYPE 2 DIABETES IN LEPTIN RECEPTOR DEFICIENT MICE (DB/DB)

Dietary saturated fat intake contributes to diabetes and cardiovascular disease, as shown in numerous animal and human studies. However, the hypothesis that stearic acid, a saturated fat, has beneficial effects on these conditions has not been adequately tested.

Leptin receptor deficient mice (db/db) and wild-type mice were fed either chow or a high fat diet enriched in either stearic acid or oleic acid for ten weeks. The progression of diabetes was evaluated with blood glucose, insulin, and metabolic parameter measurements. At the conclusion of the study, pancreatic islet organization was examined, and blood, liver and feces were assayed for fatty acid content.

The stearic acid enriched diet prevented increases in blood glucose levels independently of weight loss in db/db mice compared to an oleic acid or chow diet. Diabetic mice fed stearic acid maintained insulin responsiveness and pancreatic islet organization compared to the db/db mice fed chow and oleic diets. The islet organization of the stearic acid fed mice did not change over the course of the study and was similar to that of wild-type mice fed the same diet. Conversely, diabetic mice fed oleic acid and chow diets had decreased insulin responsiveness and disorganized islets. Stearic acid fed db/db mice had high fecal fat content and caloric intake calculations indicated low absorption of this fat.

Switching to stearic acid after prolonged hyperglycemia had a rescue effect on blood glucose levels. After feeding diabetic and wild-type mice standard chow diets for 6, 8, and 10 weeks to establish hyperglycemia, mice switched to a high fat diet enriched in stearic acid, but not one enriched in oleic acid diet, had significant reductions in blood glucose levels.

The ability of a stearic acid enriched high fat diet to slow the progression of diabetes and reverse hyperglycemia in db/db mice argues that risks and benefits of fats in the diet depend on the chemical structure, rather than the chemical class, of fats ingested. The beneficial effect of stearic acid appears to be associated with a decreased absorption of dietary fat.

Keywords: Type 2 Diabetes, Stearic Acid, Fat Absorption, Hyperglycemia Treatment, Dietary Modification

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April 26, 2012

Date

A DIET ENRICHED IN STEARIC ACID PROTECTS AGAINST THE PROGRESSION OF TYPE 2 DIABETES IN THE LEPTIN RECEPTOR DEFICIENT MOUSE MODEL (DB/DB)

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Chapter 1: Background

1.1 Diabetes and obesity

Diabetes is a widespread disease affecting an estimated 23 million Americans, and according to the American Diabetes Association, only two thirds of these cases have been diagnosed (66). The majority of cases of diabetes are type 2 diabetes. Additionally, type 2 diabetes is now a worldwide disease. The World Health Organization estimated 135 million people worldwide have diabetes in 2000, and the number of people with diabetes has been projected to reach over 300 million worldwide in the next ten years (105). The majority of new cases of type 2 diabetes will be in developing countries, and these new cases will double the current incidence of the disease (4).

The increased incidence of type 2 diabetes in developing countries begs the question, what is contributing to the onset of type 2 diabetes? One of the leading risk factors for developing type 2 diabetes is obesity. Similar to the increased incidence of diabetes, the incidence of obesity is also dramatically increasing. From 1976 to 1994, obesity in the United States has increased 8% and statistical projections predict future increases (19, 62). These increases align with the increased incidence of type 2 diabetes. Startlingly, the rate of obesity development is not greatest in adults; rather, children and adolescence have experienced the greatest rate of obesity development. Nearly 17% of U.S. children are obese and that figure has tripled since 1980 (84, 141). Globally, nearly 1.5 billion adults and over 40 million children are overweight or obese, as reported by the World Health Organization in 2008 (19). In the United States, over one-third of the adult population is obese (62). Additionally, approximately 190,000 Americans under the age of 20 have been diagnosed with type 2 diabetes and that number continues to increase.

Therefore, there is an urgent need for research in dietary contributions and modifications to halt the progression of, and lower the incidence of, type 2 diabetes worldwide.

1.2 Diet impact on diabetes

In an effort to reduce the risk of developing type 2 diabetes, many dietary recommendations have been made. Dietary components, such as carbohydrates, proteins and fats, have all been adjusted in an effort to lower blood glucose levels. The components of the diet, especially the amounts and types of fat, are major contributors to obesity and diabetes. However, a major problem still remains: overconsumption of nutrients leads to the development of obesity and type 2 diabetes. Americans are practiced in over-nutrition, too many calories and too much of each nutrient, carbohydrate, protein and fat. An average American man requires approximately 2000 calories a day to maintain normal weight and function (46). However, consuming over 2000 calories is more common than not, leading to weight gain and obesity. Additionally, these dietary calculations have not been adjusted for changes in energy expenditure (18). Most Americans are not expending 2000 calories per day and therefore should be consuming less than 2000 calories (152).

Dietary interventions have been employed throughout type 2 diabetes treatment history. In early diabetes research, the removal of carbohydrates from the diet was thought to help control blood glucose levels (87, 93, 133, 159). While this does tend to be effective for a brief time, the frank removal of a nutrient group is not sustainable. With glycemic index monitoring, different types of carbohydrates have been shown to affect blood glucose levels differently. On the basis of these observations, adjusting the diet to

incorporate more of the carbohydrates that do not adversely affect blood glucose levels has been promoted (159).

In the low glycemic index diets, such as the Zone and South Beach diets, carbohydrates are ranked according to their capability to increase blood glucose levels on a scale of 1-100 with 100 being the glucose reference point (133, 159). Low glycemic carbohydrates are more slowly absorbed so as to minimize blood glucose spikes. A few examples of low glycemic foods are whole grains, fruits and legumes; however, some low glycemic foods such as candy bars with nuts are not healthy (133). Though these foods contain low glycemic loads, they may also contain more fat. Additionally, the major outcome of these diets is not necessarily lower blood glucose, but a weight reduction by removing excess calories from the diet (59).

The Atkins diet has exploited the differential energy utilizations of protein and fat for weight loss. The Atkins diet is a low carbohydrate, high protein, moderate fat diet that has been beneficial in promoting weight loss (1, 2, 107, 113, 145, 153). Dietary components have different energy content. While carbohydrates, in the form of glucose, are the preferred energy source of our bodies, more energy can be extracted from proteins and fats than from carbohydrates. However, this increase energy extraction comes at a price. More energy is required for the metabolism of proteins and fats than for carbohydrate metabolism. The Atkins diet prescribes an elimination of carbohydrates from the diet while increasing protein intake regardless of fat content (168). The elimination of carbohydrates removes the preferred and efficient fuel source from the body. Metabolism of proteins and fats essentially burns more energy and is much slower than carbohydrate metabolism thus resulting in rapid weight loss. However, this diet has

some drawbacks. Low carbohydrate/high protein diets like the Atkins diet require the adoption of special precautions because of increased risk of raising plasma cholesterol levels (8). Additionally, the body as a system cannot be maintained on this limited diet. Most often, protein and fat are over consumed and converted to fat, thereby increasing body mass and defeating the ability of the diet to control blood glucose levels.

Diets such as the Atkins diet and the Mediterranean diet are utilized to lower blood glucose levels and body mass. The Mediterranean diet is a low fat, high fiber diet that has a low glycemic load and is rich in unsaturated fat. It promotes decreased insulin resistance and weight, both of which contribute to lower risk of developing type 2 diabetes and cardiovascular disease (144). The Mediterranean diet gained popularity after several observational studies concluded that the incidences of diabetes and coronary heart disease are lowest in Mediterranean countries (103). The Mediterranean diet is high in unsaturated fat, indicating that, in addition to the amount of fat consumed in the diet, the kind of fat consumed may be important.

A reoccurring theme throughout the literature is that the normal, healthy diet should principally contain unsaturated fats with low amounts of saturated fats and no *trans* fats (16, 17, 24, 34, 60, 67, 73, 76, 81, 82, 87, 104, 111, 124, 127, 135, 146, 151, 156, 165, 177). The recommended decrease in fat intake is beneficial in treating both obesity and type 2 diabetes by promoting weight loss. On average, a 7% or greater reduction in weight while on a fat or calorie restricted diet has been shown to decrease overall body mass and reduce diabetic symptoms, such as insulin resistance (6). The dietary modifications suggested by both the American Diabetes Association and the World Health Organization are increased dietary fiber and decreased overall fat intake,

especially minimizing saturated and trans fat intake (122). The overconsumption of dietary fats is a major risk factor in developing obesity and obesity-related diseases such as type 2 diabetes.

The current dietary treatments for type 2 diabetes, including the Atkins, Mediterranean, and South Beach diets, have depended upon elimination of a nutrient group and focused on decreasing body mass to reduce blood glucose and halt the progression of type 2 diabetes. However, these high failure rates and short lifespan of these diets makes them poor permanent solutions for the treatment of type 2 diabetes. A more focused approach by closely examining the differential effects of fats in the diet might be more effective in minimizing type 2 diabetes and its physiological consequences.

1.3 Dietary Fat

Dietary fats can be classified as either saturated, unsaturated, or trans depending on the types, placement, and origination of carbon-carbon bonds in the fatty acids (**Figure 1.3.1**). In saturated fats, each carbon is fully saturated with hydrogen and therefore the carbons have only single bonds between them (92). Unsaturated fats have at least one double bond and are named by the placement of the double bond (63). Trans fats, which rarely occur in nature, are unsaturated fats where the carbon chains extend off of the opposite sides of the double bond. Trans fats, were developed as a butter alternative in response to population growth, widespread use of refrigeration, and decreased butter supply (112). All classes of fats can vary in chain length, and this variation in chain length also alters the metabolism of fats. Long fatty acids require

additional cleavage steps, decarboxylation, to convert each long fatty acid into acetyl groups that enter the Krebs cycle during metabolism (176).

Many studies investigating the effects of dietary fat are not diet studies at all but instead are biochemical studies in cultured cell models (54, 111, 125, 142, 148, 151). Culturing cells in specific concentrations of dietary fat cannot hope to provide an accurate picture of how the body handles (metabolism and absorption) fat. These studies only tell us how the cells tolerate the concentration of fat in the culture media. The amount of fat added to the culture media may not be an accurate representation of the amount of fatty acid that is actually bathing the cells after absorption and metabolism of the fat.

This dissertation examines two dietary fats typically found in the American diet: stearic acid, a saturated fat, and oleic acid, a mono-unsaturated fat and their effects on blood glucose levels in a diabetic mouse model. The major flaw in the thought that the saturation of fat can predict the incidence of cardiovascular disease is that not all fats have been thoroughly investigated for their metabolic properties. Lumping a group of fats together based on the results given by one or two fats is not a valid venture. This dissertation examines the nutritional and metabolic differences between stearic acid, a saturated fat, and oleic acid, an unsaturated fat in diabetic and wild-type mice.

Studies of saturated fats have concluded that saturated fats increase the risk for cardiovascular disease, increase plasma cholesterol levels, and promote insulin resistance (74, 86, 162). Studies of unsaturated fats have found that unsaturated fat decreases risk for cardiovascular disease and; decreases cholesterol levels only if included in a low fat diet regimen (9, 23, 71). However, insulin resistance and blood glucose levels are

unchanged if they are included in a high fat diet, typical of the American diet (98, 174). The lack of concrete mechanistic and long-term nutritional studies on the effects of individual fatty acids in the diets of healthy subjects fuels the good versus bad fat debate. This study investigated the differential effects of one saturated fat, stearic acid, and one unsaturated fat, oleic acid, on the progression of type 2 diabetes.

1.3.1 Saturated fat

Estimates of the total content of saturated fat in the typical American diet varies from as high as 25% to as low as 12% of the total calories coming from saturated fat (10, 16, 21). In the third National Health and Nutrition examination Survey, the average saturated fat content in the diet was assessed at only 12% (5). The current National Research Council recommendation for saturated fat consumption is 10% or less of the total daily caloric intake (76, 114, 177). The progressive elimination of dietary saturated fat has driven the commercial use of hydrogenation of unsaturated fats to provide an easier and more stable cooking alternative to butter.

The lack of scientific and mechanistic understanding of the detrimental effects of saturated fat on plasma lipoprotein levels makes it possible that broad generalizations about this fat class are premature and unwarranted. Saturated fats have been labeled as ‘bad fats’ because of their association with increasing overall plasma lipoprotein levels (21, 37, 47, 48, 110, 132, 134). Therefore, only saturated fats that deleteriously effect plasma cholesterol levels have been extensively studied. The most studied dietary saturated fat is palmitic acid. However, other saturated fats have beneficial effects on plasma cholesterol levels. One of the beneficial saturated fats is stearic acid (15, 140).

1.3.2 Stearic acid

The U.S. Beef and Cattle Industry reports that beef is one of the dietary protein staples in the U.S., with over 25 billion pounds of beef consumed every year (47, 96, 134). Stearic acid constitutes roughly 25% of the total saturated fat consumed by Americans and is found in highest abundance in red meat, coconut oil, and cocoa butter (40, 47). Although stearic acid is one of the major fats in the American diet, few studies have examined the effects of stearic acid on the progression of obesity and obesity related diseases such as type 2 diabetes or the effects of dietary stearic acid on blood glucose levels, insulin sensitivity or glucose tolerance. Since stearic acid has been categorically classified as one of the detrimental fats along with all other saturated fats, it is not included in most dietary studies even though it is a major fat in the American diet (119, 171). This lack of research and the categorization of fats have left saturated fat relatively unexplored in terms of metabolic effects and therefore open to misinterpretation, especially in dietary recommendations for treatment of disease.

Since the consumption of dietary saturated fat is linked with cardiovascular incidences, many studies have evaluated the effect of stearic acid on plasma lipoproteins. The majority of these studies have found that stearic acid is beneficial in lowering total LDL cholesterol levels, but HDL and total cholesterol levels are elevated (53, 75). A few studies have found that stearic acid does not alter the plasma cholesterol profile (37, 110). A few other studies show that stearic acid lowers total cholesterol as well as LDL levels (42, 158). These results are contradictory to the current thought that all saturated fats should be avoided due to the increased total plasma cholesterol effect some saturated fats have shown. Consequently, stearic acid is considered to be a neutral or inert fat unlike

most other saturated fats (21, 72, 75, 158). The neutral or beneficial effects of stearic acid on plasma cholesterol levels are an indicator that stearic acid is not like most saturated fats and may have additional beneficial effects that can be used to treat type 2 diabetes. Stearic acid could be used as a dietary treatment for type 2 diabetes without the concern of harmful cardiovascular effects since stearic acid has neutral effects on plasma lipoproteins.

1.3.3 Unsaturated fat

Another category of dietary fats is unsaturated fat. These fats have a least one double carbon-carbon bond in the chain. Unsaturated fats have been promoted as 'good fats' because they lower LDL and increase HDL levels when compared to the effects of saturated fats (27). They have usurped many saturated fats in the diet as a preventative measure against diabetes and cardiovascular disease because of these plasma lipoprotein effects.

Though unsaturated fats are all promoted as healthy fats, there are inconsistencies and contradictions in this branch of fats as well. In diabetes research, mono-unsaturated omega-9 and the poly-unsaturated omega-6 fats have been reported to be both beneficial and detrimental for insulin resistance (3, 14, 113, 116).

Some unsaturated fats are also linked to plaque formation, a similar observation as in saturated fats. Poly-unsaturated fats have also been shown to accumulate in arteries and form atherosclerotic plaques (60). Conversely, unsaturated fats called omega-3 fatty acids, found in cold-water fish such as salmon and herring, are positively associated with reductions in atherosclerotic plaque formations and reductions in plasma cholesterol. In

diabetes, these fats are linked to reversing insulin resistance. However, like many other fatty acids, the mechanism of action is unknown.

It is interesting that in both classes of fats there are contradictions amongst the members of each class. Some saturated fats, like stearic acid have no negative effects on insulin or plasma lipoproteins, and some unsaturated fats, like the polyunsaturated fats, have no positive effects on insulin resistance or plasma lipoprotein levels.

1.3.4 Oleic acid

One dietary recommendation prescribed to type 2 diabetics is the Mediterranean diet, a moderate fat diet (~20% kcal from fat) enriched in fruits, legumes and olive oil, which provides the major fat in the diet. The major fatty acid component of olive oil is oleic acid, the unsaturated fat that health professionals have recommended to be in the diet. Oleic acid is a mono-unsaturated dietary fat that is, like stearic acid, 18-carbons in length with one double bond at the ninth carbon. Oleic acid has been reported to decrease insulin resistance (16).

Additionally, oleic acid has been shown to decrease total plasma cholesterol and low density lipoprotein (LDL) levels while increasing high density lipoprotein (HDL) levels (111). There has been no association between oleic acid and poor fatty acid absorption from the gut. The absorption of oleic acid is comparable to the absorption of palmitic acid, perhaps the most studied dietary fat (64). This positive effect on plasma lipoproteins has caused nutritionist and patient care specialists to recommend the use of oleic acid as a dietary mechanism to control or lower blood glucose levels.

1.4 Animal models of type 2 diabetes

The experimental limitations of human studies make appropriate animal models a valuable tool in identifying mechanisms of type 2 diabetes progression. Moreover, animal models are useful for developing and testing new treatments of human disease. An ideal model of type 2 diabetes is one in which the physiological aspects of human type 2 diabetes can be easily and efficiently reproduced.

The db/db mouse model is an attractive model for type 2 diabetes because the human disease can be easily and efficiently reproduced in this mouse. Hummel et al (88) first described the db/db mouse in 1966 as a model for type 2 diabetes. In this mouse model, a leptin receptor defect results in a truncated form of the leptin receptor protein (88). This truncated leptin receptor results in a hyperphagic and obese mouse, similar to human type 2 diabetes, that decreases active receptor formation, decreases ligand binding, and decreases receptor activation (115). This truncated leptin receptor is also found in humans, and though it is not the cause of the majority of diabetic cases, serves as an efficient, malleable model of human type 2 diabetes (61).

Leptin is an adipocyte secreted protein that regulates satiety in the hypothalamus of the brain. After a meal, leptin is secreted from the adipocytes and feeds back onto the satiety pathway stopping the urge to eat. Without leptin, the negative feedback system is lost, and the animal eats insatiably and gains weight.

Type 2 diabetes progresses quickly in the db/db mouse model. As early as ten days of age, db/db mice have increased insulin secretion and moderate hyperglycemia. Plasma insulin levels continue to increase for three months as beta cell mass increases in an attempt to compensate for the increased severity of hyperglycemia (61). A drop in

insulin to near normal levels may occur after three months of age as the beta cells begin to atrophy and die (61). Plasma glucose levels increase to over 400 mg/dl, a level that is maintained throughout the lifespan of the mouse leading to an abbreviated lifespan compared to the wild-type mouse (38).

1.5 Statement of Research

Overconsumption of the diet is widespread and leads to obesity and type 2 diabetes. High dietary fat is one of major contributors to obesity and type 2 diabetes. Some nutritional interventions have aimed at decreasing carbohydrate and fat content in the diet in an effort to lower body mass and blood glucose levels. However, the success rate and longevity of these diets are low. Previous nutritional studies have shown that saturated fat increases the risk for cardiovascular disease as well as type 2 diabetes and additionally have shown that low fat diets enriched in unsaturated fats, such as oleic acid, could improve insulin resistance, lower body mass and blood glucose levels. The current American Diabetes Association recommendations of a low fat diet containing mostly unsaturated fats and exclude most saturated and all trans fats. These past studies have done little to examine the effects of individual fatty acids on weight and blood glucose levels.

The human diet is composed of a mixture of fats making it challenging to draw conclusions about the impact of any particular fatty acid on the progression of disease states such as diabetes. Therefore, more studies are necessary to evaluate the effects of specific fatty acids, such as stearic and oleic acids, on diabetes and heart disease. Most of what is known about the functions of fatty acids is fragmented and biased by the assumptions made within the experimental investigations in which the fatty acids were

studied. This bias is particularly true for studies of the saturated fatty acids, most of which have been examined solely for their tendency to alter lipoprotein metabolism and to influence the concentrations of lipoproteins that carry cholesterol in blood.

Stearic acid is one of the three most common saturated fats in the diet, constituting roughly 25% of the total saturated fat consumed by Americans. It is found in highest abundance in red meat, coconut oil and cocoa butter (40, 47). Since stearic acid has been categorically classified as one of the detrimental fats, few studies have examined the specific effects of stearic acid on blood glucose levels or insulin sensitivity (55, 123). In cardiovascular research, stearic acid has no effect on plasma cholesterol levels (75). Consequently, stearic acid is considered to be a neutral or inert fat, unlike most other saturated fats (21, 72, 75, 158).

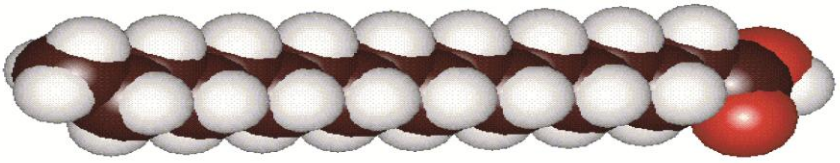
For this study, high fat diets were designed to mimic Western diets that typically contain at least 35% of their caloric value from fat; the experimental diets contained 40% of the total kilocalories from fat. For each experimental diet, the majority of the fat content was either in the form of stearic acid (85% of total fat) or oleic acid (67% of total fat). These diets were fed to db/db mice and wild-type mice for ten weeks.

Aim 1: **Determine if a high fat diet enriched in stearic acid slows the progression of type 2 diabetes in diabetic (db/db) mice.** High fat diets enriched in saturated fat have been labeled as detrimental to overall health and allegedly linked to elevated incidence of cardiovascular events. However, the saturated fat stearic acid has no effect on plasma cholesterol levels unlike the saturated fat palmitic acid, which increases LDL cholesterol. If stearic acid has beneficial effects on type 2 diabetes, it could be included in the diet without detrimental cardiovascular effects.

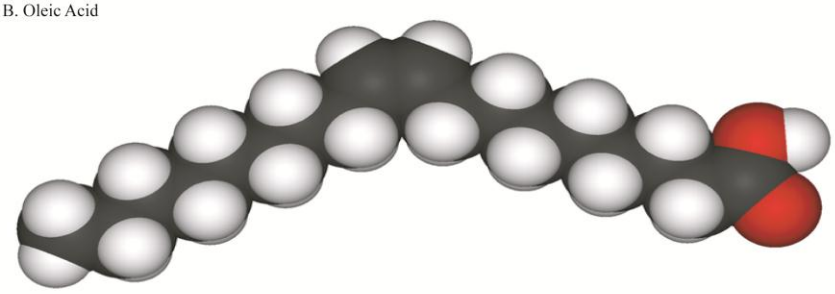
Aim 2: **Determine the appropriate dietary dose to get a beneficial effect of decreased blood glucose from stearic acid in db/db mice.** A high-fat diet has clinical complications other than type 2 diabetes, such as cardiovascular disease and obesity. While the high fat diet enriched in stearic acid may have beneficial effects, it is unlikely that nutritionists and patient care specialists will recommend a high fat diet to patients. Additionally, the U.S. Department of Agriculture recommends a diet containing less than 30% fat. Therefore, lowering the fat content in the diet to 17% of the total kilocalories from fat may be a more viable and attractive dietary intervention for the treatment of type 2 diabetes. I created and tested a moderate fat diet enriched in stearic acid in order to evaluate the if stearic acid in a moderate fat diet had the same beneficial effects on blood glucose levels as the high fat diet enriched in stearic acid.

Aim 3: Determine if a high fat diet enriched in stearic acid will lower blood glucose levels in diabetic mice with prolonged and untreated hyperglycemia. In humans, diabetes is not treated until after clinical symptoms, such as hyperglycemia, have been present, sometimes for years; therefore, the ability to rescue the normal blood glucose phenotype by diet modification would be advantageous.

A. Stearic Acid



B. Oleic Acid



C. Trans Fat (Eladic Acid)

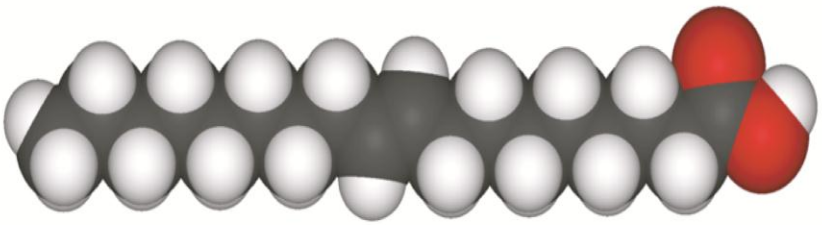


Figure 1.3.1: Typical Dietary Fat Models.
A. Stearic acid is an eighteen carbon saturated fat that is most abundant in red meat and chocolate. **B.** Oleic acid is an eighteen carbon unsaturated fat found in olive oil. **C.** Trans fats can be long or short chained unsaturated fats where hydrogens bound to the carbons at the double bond extend from the same side of the double bond. Red spheres are oxygen.

Chapter 2: A High Fat Diet Enriched in Stearic Acid Slows the Progression of Type 2 Diabetes in db/db Mice

2.1 Introduction

A diet high in fat contributes to the progression of type 2 diabetes (24, 137, 151). Most diabetic nutritional studies have focused on general classes of dietary fat, saturated versus unsaturated, but have left any differential effects of individual fatty acids relatively unexplored (135, 142, 151). Previous studies reported that low fat diets enriched in unsaturated fats have beneficial effects on insulin sensitivity and glucose tolerance, but diets enriched in saturated fats have detrimental effects on insulin sensitivity and glucose tolerance (50, 54, 171). In contrast, all high-fat diets, other than n-3 fatty acids, reportedly led to insulin resistance (165). These data suggest that the effects of dietary fats on the progression of type 2 diabetes can be predicted simply from the degree of saturation of ingested fat.

In cardiovascular research, individual fats have been studied for their effects on cholesterol profile and progression of cardiovascular disease. Saturated fats have, in fact, been labeled as ‘bad fats’ because of their association with increasing overall cholesterol and LDL levels (21, 37, 47, 48, 110, 132, 134). Conversely, unsaturated fats have been promoted as ‘good fats’ because they lower LDL and increase HDL levels when compared to the effects of saturated fats. They have usurped many saturated fats in the diet as a preventative measure against diabetes and cardiovascular disease. In particular, oleic acid, an 18-carbon mono-unsaturated fatty acid, lowers LDL and raises HDL levels when compared to the saturated fats palmitic acid and myristic acid (16). In the Western diet, at least 35% of the calories are from fat. The American Heart Association

recommends no more than 30% of calories be from fat in the diet (108). Given that positive orosensory feedback of dietary fat consumption works against efforts to reduce fat consumption, identifying specific fats that are inert or even beneficial in terms of type 2 diabetes and cardiovascular disease would offer promising alternatives for nutritional intervention (39).

Stearic acid is one of the three most common saturated fats in the diet, constituting roughly 25% of the total saturated fat consumed by Americans. It is found in highest abundance in red meat, coconut oil and cocoa butter (40, 47). Since stearic acid has been categorically classified as one of the detrimental fats, few studies have examined the effects of stearic acid on blood glucose levels or insulin sensitivity (55, 123). In cardiovascular research, stearic acid has no effect on plasma cholesterol levels (75). Consequently, stearic acid is considered to be a neutral or inert fat, unlike most other saturated fats (21, 72, 75, 158). In this study I hypothesize that the 18-carbon saturated fat, stearic acid, has beneficial effects on the progression of diabetes.

For this study, high fat diets were designed to mimic Western diets; the diets contained 40% of the total kilocalories from fat. The majority of the fat content was either in the form of stearic acid (85% of total fat) or oleic acid (67% of total fat). These diets were fed to db/db mice and wild-type mice for ten weeks. Db/db mice fed a high fat diet enriched in stearic acid had lower blood glucose levels than db/db mice fed either the high fat diet enriched in oleic acid or the standard chow diet. These effects on glucose were independent of weight loss. Instead, the stearic acid diet was associated with reduced fat absorption and as a consequence metabolic switches from fat to protein and carbohydrates for calories and energy derivation.

2.2 Materials and Methods

2.2.1 Animals

Initial cohorts of wild-type, age-matched, male C57BLKS/J (WT, n=105) and BKS.Cg-*Dock7*^{m} +/+ *Lepr*^{db}/J (db/db, n=105) mice were purchased from The Jackson Laboratory (Bar Harbor, Maine) at four weeks of age. Upon receipt they were acclimated for one week to baseline conditions of a 12-hr light/dark cycle at 25°C on an *ad libitum* diet of commercially available rodent chow diet (2018 Teklad Global 18% Protein Rodent Diet; Harlan Laboratories, Madison, Wisconsin). At five weeks of age, I weighed the mice, gave each an ear tag, drew blood for a baseline blood glucose measurement, and randomly assigned each mouse to a diet group (n=5 mice per group per genotype). The diets were the baseline chow diet and two experimental diets (40% kcal stearic acid diet Harlan Teklad TD.04096, 40% kcal oleic acid diet TD.09055). Mice were fed *ad libitum* for 10 weeks. Metabolic measurements were performed every two weeks through the duration of the study (**Figure 2.2.1**). About one year later a second cohort of mice (n=10 animals per diet per genotype) was treated identically and used to make additional metabolic and food consumption measurements. Weight, blood glucose and insulin tolerance measurements were made only at the start of the diet and at the endpoint in this cohort of animals. A rescue study was designed with aged WT (n=16) and db/db mice (n=16) in a third, and final cohort. These mice were purchased from The Jackson Laboratory at 5 weeks of age and fed chow diet until 10 weeks of age. The mice were then switched to 40% stearic diet (n=8 per genotype) or 40% oleic diet (n=8 per diet group) and fed *ad libitum* for 6 weeks. Blood glucose, insulin tolerance and weight were measured at the start of diet and after 6 weeks on diet. Animal care and housing were

conducted according to the NIH *Guide for the Care and Use of Laboratory* Animals. All experiments were approved by the Institutional Animal Care and Use Committee at the University of Kentucky.

2.2.2 Diets

Tables 2.1, and **2.2** display the nutritional value and composition of each diet used in this study. The stearic and oleic acid diets used in this study contained similar percentages of protein and carbohydrate whereas chow diet contained slightly more of total kcal from protein and 60% total kcal from carbohydrates. The fat content of each diet was unique. The experimental diets were custom made by Harlan Teklad using a modified TD.03459 diet. A Harlan Teklad nutritionist calculated all dietary nutritional values.

2.2.3 Food Consumption

Study diet (200g) was placed into the food manger of each cage. The remaining food was measured and replaced every 48 hours. Food consumption was calculated from the starting food and remaining food weights. Caloric and fat intakes were calculated from food intake data and caloric and fat values of specific diet. In a second study, metabolic monitoring cages were used to measure food consumption from a hanging food cage over a two-day period.

2.2.4 Glucose Measurements and Insulin Tolerance Test

Insulin tolerance tests (ITT) were performed in the first cohort of mice every two weeks on five mice per diet group per genotype. Mice were weighed and then fasted for four hours in a clean cage prior to testing. Fasting blood glucose was measured by tail

prick just prior to insulin injection and at the times indicated in “Results” using a commercially available glucometer and test strips (One Touch Ultra Glucose Monitoring Kit, Lifescan, Milpitas, California). Mice were injected (i.p.) with human insulin (0.2U/g) (Lilly, Indianapolis, Indiana).

2.2.5 Insulin ELISA

Blood collected from fasted animals (4 hour fast) was allowed to clot for 20 minutes in a vacutainer, and then centrifuged at 1500 X g for 10 minutes to isolate serum. Serum was then snap-frozen and stored at -80°C until analyzed. Plasma insulin levels were measured using a commercially available mouse/rat insulin ELISA kit (Millipore, Billerica, MA) and reported in ng/mL.

2.2.6 Body Composition

Body composition, including fat mass and lean mass, was determined using EchoMRI Quantitative Magnetic Resonance Body Composition Analyzer (Echo Medical Systems, Houston, Texas) on conscious mice (n=5 per diet per genotype). Conscious mice were placed into the measuring tube and the tube placed into the EchoMRI machine. The EchoMRI uses the distinctions in NMR amplitude signals of various tissues to determine mass of muscle, fat and body fluids.

2.2.7 Oxygen Consumption

Five mice per diet group per genotype were acclimated to the oxygen consumption chambers for 30 minutes prior to indirect calorimetry measurements. Metabolic rate was indirectly determined using weight, temperature and oxygen consumption. Conscious mice were weighed and placed in oxygen consumption

chambers. Room air was pumped into and out of the chambers and analyzed for oxygen and carbon dioxide content (mL/hr) using a CWE metabolic monitoring system and software (CWE, Allentown, Pennsylvania). Oxygen consumption was then computed from these measurements using the equation $\dot{V}$O2/weight (mL/g/hr) .

In a separate experiment, five db/db mice per diet group from the second cohort were acclimated in individual indirect calorimetry monitoring cages (TSE Labmaster Metabolism Research Platform, Midland, Michigan) with free access to experimental diet and water for three days during week ten of the diet study. After the acclimation period, complete metabolic data including activity level, food consumption, and water consumption were collected for 48 hours. From these measurements, activity levels during the mouse active period (18:00-06:00) were isolated to make resting metabolic rate calculations using the formula (160):

$$RMR = \frac{\left(3.941 * \frac{FlowML * (V1+V2)}{N2Ref * Animal\,Weight * 100}\right) + \left(1.106 * \frac{FlowML * (dCO2)}{Animal\,Weight * 100}\right)}{1000}$$

2.2.8 Immunohistochemistry

After all measurements were made, all animals in each group of mice (five mice per group per genotype; Figure 2.2.1) were anesthetized (3% isoflurane), euthanized by thoracotomy, and blood was collected by heart puncture. Note, therefore, that an individual group was tested every two weeks of the study. Animal tissues were perfused with 10ml PBS (pH7.4) followed by perfusion fixation with 10mL of formalin (4% paraformaldehyde). Tissues were harvested, placed in formalin overnight at 4^0C, and embedded in paraffin wax. Sections (5µm thickness) of each tissue were cut using a microtome and placed on PermaFrost glass slides. Sections were then de-paraffinized and used for immunohistochemistry. The following primary antibodies were used for insulin

and glucagon staining: guinea pig polyclonal anti-human insulin (1:50) and rabbit anti-glucagon (1:250). The secondary antibodies were Cy3 donkey anti-guinea pig (1µg/ml) and Cy5 goat anti-rabbit (1µg/ml). All antibodies were purchased from Jackson Immuno Research (West Grove, Pennsylvania). Slides were processed for microscopy and images were taken at 20X magnification (Nikon Eclipse-Ti microscope, Melville, New York). The exposure time for anti-insulin was 300ms and for anti-glucagon was 3secs. Nikon NES Elements software was used to process images. Islet area was calculated by tracing the outline of each islet using the NES Elements software. For cell density calculations, cells with positive DAPI staining were counted as viable cells. Insulin-positive cells where counted as beta cells and glucagon-positive cells were counted as alpha cells. Cell density (total cell density, beta cell density, and alpha cell density) was calculated as total number of positive staining cells/area of the islet (μm^2). Alpha to beta cell ratio was calculated by total positive alpha cells/total positive beta cells per islet. Alpha cell migration was calculated by measuring the distance from the alpha cell to the outer edge of the islet and normalized to the length of the radius (using the geometric center of the islet section) passing through each cell.

2.2.9 Gas Chromatography/Mass Spectrometry

Tissue, serum, and feces were collected post-mortem every two weeks during the study. Total fatty acids were extracted from 100mg of samples using Folch method. Extractions were then analyzed using GC/MS. Briefly, a 1 µl sample was injected into the gas chromatography system (model 6890GC G2579A system; Agilent, Palo Alto, California) equipped with a column (J&W DB5HT capillary columns, Agilent Technologies) and a flame ionization detector. An Agilent 5973 network mass selective

detector was used to identify target peaks of individual fatty acids. First, the corrected peak area of the FA of interest in the sample was calculated by multiplying by the ratio of peak area of the internal standard in standard set and the peak area of the internal standard in the sample and then multiplying by the ratio of the internal standard concentration in standard set and sample. Second, the concentration of the FA of interest was calculated by multiplying the corrected area of the interest peak by the ratio of the interest FA in the standard and in the sample. The two-step formula used is below.

$$\mathrm{A_{c,x}} = \mathrm{A_x} * \left(\frac{\mathrm{A_{C17,standard}}}{\mathrm{A_{C17,sample}}}\right) * \left(\frac{\mathrm{CONC_{C17,sample}}}{\mathrm{CONC_{standard}}}\right)$$

$\mathrm{A_x}$ denotes the peak area of the fatty acid of interest in the sample. The area of the peak is corrected for sample variation and $\mathrm{A_{c,x}}$ denotes the corrected peak area of the fatty acid of interest in the sample. $\mathrm{A_{C17,standard}}$ and $\mathrm{A_{C17,sample}}$ represent the area of the internal standard in the standard solution and in the sample. Next, the sample concentration was calculated.

$$CONC_x = A_{c,x} * \left(\frac{CONC_{x,standard}}{A_{x,standard}}\right)$$

2.2.10 Data Analysis

Values are depicted as mean $\pm$ standard error and considered significant if $p < 0.05$. Data were statistically analyzed using two-way ANOVA with Bonferroni correction or one-way ANOVA with Dunnett's post hoc test to identify which means differed using GraphPad Prism 5.01 for Windows (GraphPad Software, San Diego, California). NIS-Elements 3.0 (Nikon Instruments, Elgin, Illinois) was used for finding islet area and cell counts.

2.3 Results

2.3.1. A high fat diet enriched in stearic acid limits the progression of hyperglycemia in db/db mice.

Fasting blood glucose levels were determined at the initiation of high fat diet feeding (week 0 baseline) and every two weeks during the ten week study for both WT and db/db mice. Data shown in **Figure 2.3.1A** are for baseline and measurements taken after 10 weeks on diet. For WT mice on chow or oleic acid diet the blood glucose levels did not change throughout the study. In contrast, WT mice fed stearic acid diet had significantly decreased fasting blood glucose beginning at two weeks on diet (not shown, Figure 2.3.1A). The baseline glucose levels for db/db mice were about two-fold higher than WT mice, as expected (38). Groups of db/db mice fed chow or oleic acid approximately doubled their blood glucose levels in ten weeks. In marked contrast, db/db mice fed stearic acid did not have an increase in blood glucose levels.

This experiment was repeated with a second cohort of animals. After ten weeks on diet, wild-type and db/db mice fed a high fat diet enriched in stearic acid had significantly lower blood glucose levels than the corresponding chow fed or oleic acid fed mice (**Figure 2.3.1B**). The db/db animal fed stearic acid also had blood glucose levels significantly lower than their baseline measurements. The db/db mice fed chow and a high fat diet enriched in oleic acid had significant increases in blood glucose from baseline measurements after 10weeks on diet.

2.3.2. Mice fed a high fat diet enriched in stearic acid have lower blood glucose after only two weeks on diet.

After only 2 weeks on the high fat diet enriched in stearic acid, the db/db mice fed this diet had lower blood glucose levels than the db/db mice fed either oleic or chow diets (**Figure 2.3.2**). Decreases in blood glucose after diet intervention usually correspond to a decrease in body weight as well (45, 58, 101, 117, 129). This quick response to the diet has been reported in other diet interventions and may be explained by the gut flora adjusting to the new diet (32, 43, 78). The db/db mice on the stearic acid diet had an initial and abrupt decrease in blood glucose after 2 weeks on diet. However, the db/db mice fed stearic acid diet had slow and steady increases in blood glucose starting after 4 weeks on diet.

2.3.3. Body compositions of mice after 10 weeks on diets.

In diabetic models, low blood glucose levels are usually linked to decreases in weight (41, 59, 83). However, the first cohort of diabetic mice placed on high fat diets, gained weight over the ten week study (**Figure 2.3.3A**). The db/db mice fed oleic acid gained significantly more fat mass than the other db/db mice (**Figure 2.3.3C**) with no significant changes in lean mass (**Figure 2.3.3B**). WT mice fed chow diet gained lean mass from baseline (**Figure 2.3.3B**) while those fed oleic diet gained fat mass from baseline (**Figure 2.3.3C**). Importantly, db/db mice fed stearic acid also gained weight over the 10 week study; therefore, their decreased blood glucose levels were not caused by weight loss. Recall that db/db mice fed the stearic acid diet had lower glucose levels than mice fed the oleic acid diet. These mice also maintained a stable weight over the

course of the experiment (**Figure 2.3.3D**), confirming that the effects of stearic acid on blood glucose levels are not dependent on weight loss or gain.

2.3.4. A high fat diet enriched in stearic acid does not promote weight loss in db/db mice.

Normal diet interventions in diabetic models typically use weight loss as a guide of diet performance. Large weight loss usually corresponds to a decrease in blood glucose levels and an overall improvement in health. Interestingly, in this diet study, the db/db mice that were fed a high fat diet enriched in oleic acid, an unsaturated fat, gained a significant amount of weight over the course of the diet study as did the db/db mice fed chow diet (**Figure 2.3.4**). The db/db mice fed the oleic acid diet gained a significant amount of weight from baseline (start of the diet). Even more interestingly, the db/db mice fed a high fat diet enriched in stearic acid, a saturated fat, did not gain a significant amount of weight over the course of the ten week diet study. These mice did weigh more than their wild-type counterparts indicating that they were still obese mice; however, unlike the db/db mice fed high fat diet enriched in oleic acid, the stearic acid fed mice were able to maintain a consistent weight for the duration of the study, with one exception: the db/db mice fed stearic acid did experience an initial weight loss after 4 weeks on diet, but all mice regained the lost weight and more, becoming indistinguishable in weight from mice fed chow and oleic acid diets.

2.3.5. Diabetic mice fed a high fat diet enriched in stearic acid had lower metabolic rates than chow fed diabetic mice.

Increases in resting metabolic rate or in activity could result in lower blood glucose levels without concomitant weight loss. To assess these alternate explanations, I

used indirect calorimetry cages and calculated resting metabolic rate from the oxygen consumption and activity measurements of db/db mice after 10 weeks. Db/db mice fed oleic acid diet had resting metabolic rates no different than chow fed db/db mice and significantly higher than stearic acid fed db/db mice (**Figure 2.3.5**). In fact, the db/db mice fed stearic acid had lower resting metabolic rate than both the oleic and chow fed db/db groups. That is, stearic acid did not increase resting metabolic rate in db/db mice, but instead lowered the resting metabolic rate of these mice. Therefore, stearic acid did not affect the blood glucose levels of these mice by increasing the metabolic rate.

2.3.6. Stearic acid fed db/db mice had no differences in activity level compared to chow fed animals.

Since db/db mice fed stearic acid had lower metabolic rates than the db/db mice fed chow and oleic acid diets, I examined the activity level, one of the variables in metabolic rate, from the stearic acid fed mice while in the calorimetric cages. I found that the activity levels of db/db mice fed the stearic acid diet did not differ from those of the db/db mice fed the chow and oleic acid diets (**Figure 2.3.6**). Therefore, decreased activity was not the explanation for the lower metabolic rate seen in the db/db mice fed a high fat diet enriched in stearic acid.

2.3.7. Mice fed a high fat diet enriched in stearic acid consumed less food than chow or oleic acid diet groups.

Another factor that could affect blood glucose is reduction in food consumption compared to baseline or across dietary groups. Interestingly, db/db mice fed the high fat diet enriched in stearic acid consumed less food than db/db mice fed either chow or oleic acid-enriched diet (**Figure 2.3.7**). Although the db/db mice fed the stearic acid diet

consumed less food, the amount consumed was sufficient to maintain their weight equivalent to wild-type mice. These data suggest that even though calories consumed were sufficient to maintain a normal weight, db/db mice must have consumed less fat.

2.3.8. db/db mice absorb dietary stearic acid poorly.

Another means by which stearic acid might contribute to reduced fat load is if stearic acid were poorly absorbed from the gut as compared to other fats. I therefore examined the fatty acids excreted in the feces. Db/db mice fed the stearic acid-enriched diet excreted substantial amounts of stearic acid in the feces while mice fed oleic acid did not show increased excretion of oleic or stearic acid in the feces (**Figure 2.3.8**). Unlike oleic acid, the stearic acid was poorly absorbed by the mice. These findings argue that mice fed the stearic acid diet not only ingested fewer calories but also obtained a lower percentage of calories from fat than mice fed the other diets.

2.3.9. A high fat diet enriched in stearic acid does not cause an increase in fat accumulation in the liver.

Given that less fat was absorbed from the stearic acid diet, then these mice should show less accumulation of fatty acid in the liver, a major site of fatty acid accumulation in diabetic mice. The db/db mice, regardless of diet, had greater accumulation of fatty acid in the liver than wild-type mice ($p<0.05$, $n=5$ per diet group per genotype), as has been previously documented for db/db mice (147). However significant additional fatty acid accumulation in the liver over the 10 week study only occurred in db/db mice fed oleic acid, and only oleic acid, not stearic acid, increased in the livers of these mice (**Figure 3.3.9**). A diet enriched in stearic acid did not contribute to an excess accumulation of fat in the liver which is consistent with the observation that these mice

had lower absorption of dietary stearic acid from the gut than db/db mice fed either the chow or oleic diets.

2.3.10. A high fat diet enriched in stearic acid did not diminish insulin responsiveness.

A reduction of fat absorption and lower blood glucose may also indicate improved insulin tolerance. Diets rich in unsaturated fat, especially oleic acid, and low fat diets decrease insulin resistance in db/db mice (16, 136, 154, 163, 170). After 10 weeks on diet, WT mice had no change from baseline in insulin response (**Figure 2.3.10A** and **C)**. Though db/db mice fed stearic acid diet seemed to have insulin tolerance similar to baseline (**Figure 2.3.10B**), when plotted as a percent of fasting blood glucose measurement, the effect was not maintained (**Figure 2.3.10D**). Similarly, no change in insulin tolerance was observed in mice fed the chow and the oleic acid diets.

2.3.11. A high fat diet enriched in stearic acid did not alter plasma insulin content.

Since db/db mice fed a high fat diet enriched in stearic acid did not have decreased insulin sensitivity, I hypothesized that the pancreatic islet organization in these animals would be normal. Moreover, these animals should be spared from any pancreatic apoptosis that has been associated with the progression of type 2 diabetes (34, 35, 57, 127). As predicted, I observed no differences in plasma insulin levels in any of the diet groups after ten weeks on diet (**Figure 2.3.11**). These findings argue that pancreatic islets in the mice fed stearic acid diet would be spared the apoptotic loss of beta cells and the subsequent disorganization that normally accompanies the progression of type 2 diabetes in this model (34, 57, 127).

2.3.12. A high fat diet enriched in stearic acid protected the pancreatic islets of db/db mice from disorganization.

Pancreatic islets of WT mice appeared normal after 10 weeks on high fat diets (**Figure 2.3.12 A, C, E, G)**. In contrast, db/db mice fed the chow and oleic acid diets for ten weeks had evidence of islet disorganization (**Figure 2.3.12 B, D, F, H**). Specifically, the alpha cells, which normally lie in the outer ring of the islet, invaded the inner mass of the islets. Additionally, the islets' cores had more areas devoid of insulin and/or glucagon staining, consistent with the beginnings of β cell loss that is characteristic of advanced type 2 diabetes (99, 118). In contrast, db/db mice fed stearic acid diet for ten weeks had normal islet organization. Beta cell to alpha cell ratio was maintained in the db/db mice fed stearic acid but was disrupted in the db/db mice fed chow and oleic acid diets (**Figure 2.3.12 C**).

2.4. Discussion

Our results indicate that mice fed a high fat diet enriched in stearic acid had lower blood glucose levels and normal appearing pancreatic islets compared to mice fed other diets. These effects were not dependent on weight loss or altered resting metabolic rate. Instead, the beneficial effects of stearic acid were associated with poor absorption of stearic acid from the gut. That these mice were able to increase or maintain body weight argues that the mice must have increased utilization of the other energy sources in the diet, carbohydrates and proteins. More broadly, the benefits of a diet in which the fat content is primarily stearic acid, a saturated fat, argue that expecting all saturated fats in the diet to have the same effects is not justified. Similarly, the detrimental effects of the oleic acid-enriched diet which I have documented argue that overconsumption of this

unsaturated fat, whose consumption has been promoted due to benefits for cardiac health, elevates risk of diabetes.

Several animal studies have shown that stearic acid has lower absorption from the gut than other fats (25, 91, 134), yet another study found no differences (20). The resolution of these conflicting data is the mode of fat delivery; only when liquid fat diets were delivered orally or by infusion was absorption of stearic acid comparable to other fats. I used stearic acid in its natural solid fat form in our diet. To maximize the translatability of this study, I chose a normal oral delivery route involving stearic acid-enriched food pellets.

Human dietary studies with stearic acid have resulted in a wide range of absorption values. These discrepancies may be due to the amount and type of stearic acid in the diet, the length of the study, and the method used to detect absorption. In a recent study, Baer and colleagues (10) reported lower absorption of stearic acid as compared to palmitic acid and myristic acid by examination of fecal fatty acid content after feeding diet for two weeks. This study, similar to our study, included mixed fat diets with higher percentages of experimental fats and followed male participants over time to allow for adjustments to the diet. Conversely, Bonanome and Grundy (22) evaluated fatty acid incorporation into chylomicrons after one fatty meal and found no difference in stearic acid incorporation into chylomicrons as compared to palmitic acid. This snapshot, while useful for determining effects of stearic acid in plasma lipid profile, does not offer insight into long-term weight maintenance and glucose homeostasis. My study offers a new perspective on dietary fat intake and the progression of diabetes by using a long-term diet scheme and additional measures of metabolic parameters.

In this study, I found that mice fed a diet enriched in stearic acid had decreased food consumption. This decrease in food consumed may be due to the poor palatability of stearic acid in mice. Most fat in food is in the form of triglycerides. However in this diet study, we enriched the stearic acid diet with pure stearic acid, a free fatty acid. Fat palatability is linked to the ability of the fatty acid to activate fatty acid receptors, CD36 and GPR120, on tongue (65, 68, 102) . Saturated fats have a low affinity for these fatty acid receptors therefore the food enriched in saturated fat would be unpalatable to the mice (85, 130). Previous studies have shown that the palatability of fatty acids differs, and mice prefer unsaturated long-chain fatty acids over saturated long-chain fatty acids (155, 175). Additionally, high concentrations of fatty acids do not occur naturally in any food; therefore mice never experience them. This is not to say the same system is not present it humans. However, humans are exposed to higher concentrations of fatty acids in foods than mice and may utilize more than tongue fatty acid receptors to determine palatability. Therefore, a high fat diet enriched in stearic acid, though causes a decrease in food consumption in mice, may not have the same orosensory effects in humans.

The ability of the stearic acid-enriched diet to slow, and even reverse, diabetic symptoms is similar to some more extreme diets. Fat and caloric restriction diets have been reported to lower blood glucose levels and improve insulin response in humans with type 2 diabetes. These interventions have worked by lowering the overall caloric content and glycemic load of the diet (49, 73, 76, 81-83, 94, 116, 126, 131, 149, 156, 161, 172). This similarity in outcomes lends additional support to the conclusion, driven by my evidence of poor absorption of stearic acid, that the stearic acid-enriched diet was effective for the same reason: shifting caloric utilization away from fats to carbohydrates

and proteins. However, the stearic acid diet achieved the same blood glucose lowering effect without having to restrict calories available to the mice. Neither did it force the mice to lose weight, a property unlike dietary modifications currently used for treating type 2 diabetes consists (3, 12). My data argue that weight loss is not the exclusive key to blood glucose management, but that a nutrient shift from fat to carbohydrates and proteins in the diet slows the progression of type 2 diabetes in the absence of weight loss.

As the data have demonstrated, the existing generalizations about saturated and unsaturated fats in the diet are not appropriate. I found that the effects of stearic acid greatly differ from those palmitic acid and myristic acid, more widely studied saturated fats. Consumption of a diet rich in stearic acid slowed the progression of type 2 diabetes. Conversely, consumption of palmitic acid and myristic acid has been shown to impair insulin sensitivity (139, 150). Similar differences among saturated fats are found in studies of cardiovascular disease. Palmitic acid, the most abundant saturated fatty acid in the diet, detrimentally increases total plasma cholesterol levels (166), but myristic acid, a less common dietary saturated fat, beneficially raises HDL cholesterol (55, 121). Stearic acid is dissimilar from both of these saturated fats; it does not alter plasma cholesterol levels (21, 37, 91, 110).

Generalizations about unsaturated fats may also be inappropriate. Unsaturated fats have been promoted in the diet to replace saturated fats. However, if direct substitution is not accompanied by a reduction in overall fat intake, the overconsumption of these 'good' fats may contribute to obesity and type 2 diabetes. I found that a high fat diet that consists mainly of oleic acid was not beneficial for lowering elevated blood glucose levels or decreasing weight in the obese mice. In fact, this mono-unsaturated fat enriched

diet accelerated the progression of diabetic symptoms in db/db mice. Our data argue that although unsaturated fats have benefits for cardiovascular health, their overconsumption may increase risk for type 2 diabetes.

In a diabetic mouse model, a high fat diet enriched in stearic acid prevented an increase in blood glucose and maintained pancreatic islet organization independent of weight loss. These effects were associated with poor stearic acid absorption. Overall our data suggest that the benefits conferred from the stearic acid may have resulted in enhanced metabolic utilization of dietary carbohydrates and proteins instead of fat. Additionally, our data suggest that current dietary stands and generalizations about fat may be inappropriate and even detrimental in treating type 2 diabetes.

Table 2.1: Dietary Compositions

	Catalog Number	Protein	Carbohydrate	Fat
Chow	TD. 2918	18.80	53.80	17.0
40% kcal Stearic Acid	TD. 04096	17.80	42.20	40.0
40% kcal Oleic Acid	TD. 09088	17.50	41.50	41.0

Table 2.2: 40% Diet Fat Composition

Diet	Chow	Stearic Acid	Oleic Acid
% kcal from Fat	17.00	40.00	41.00
% Stearic acid in fat	2.60	86.00	2.00
% Oleic acid in fat	22.50	<1.00	64.00
% Other Essential Fatty Acids	74.90	13.00	34.00

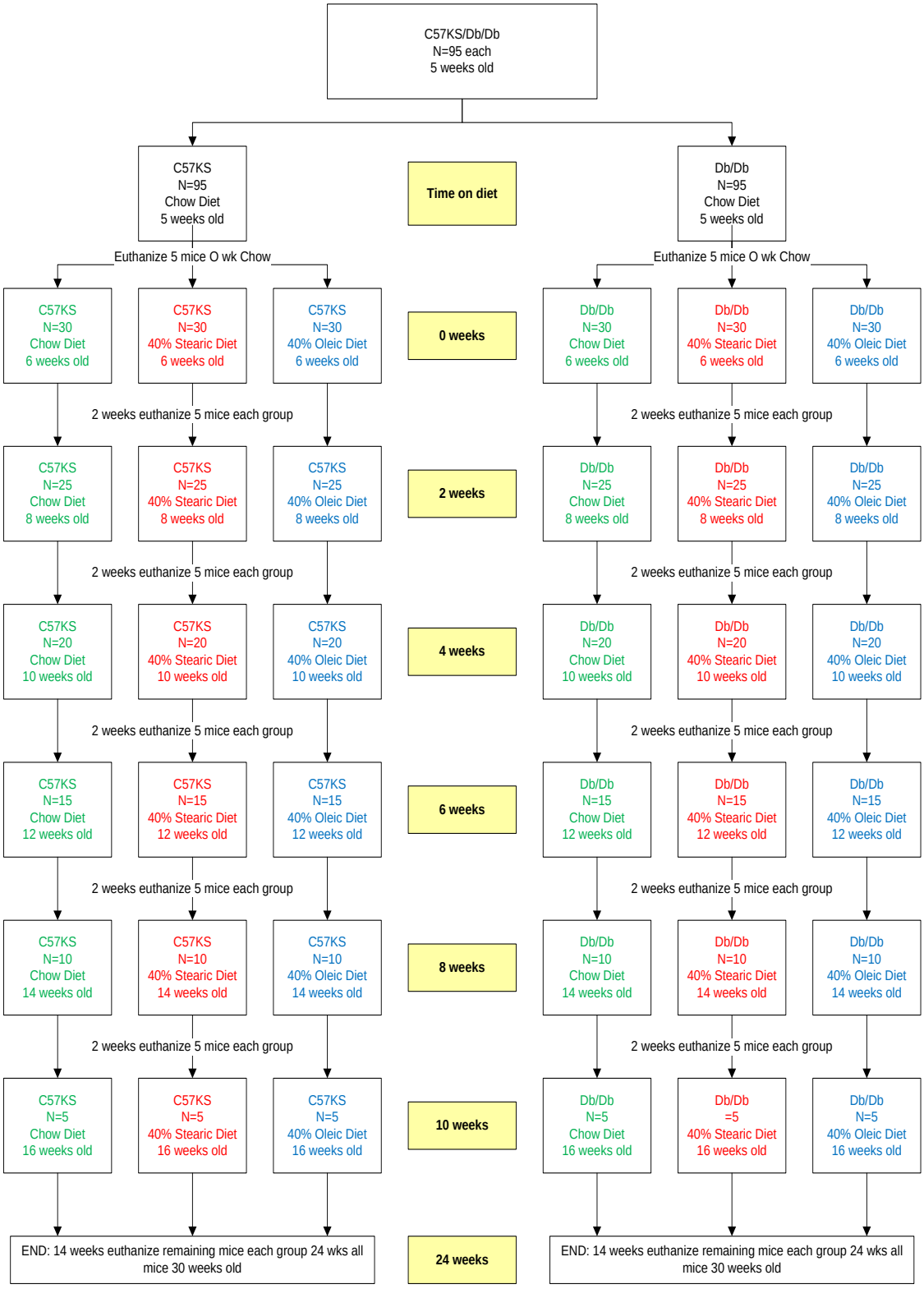

C57KS/Db/Db
N=95 each
5 weeks old
C57KS
N=95
Chow Diet
5 weeks old
Time on diet
Db/Db
N=95
Chow Diet
5 weeks old
Euthanize 5 mice O wk Chow
Euthanize 5 mice O wk Chow
C57KS N=30 Chow Diet 6 weeks old
C57KS N=30 40% Stearic Diet 6 weeks old
C57KS N=30 40% Oleic Diet 6 weeks old
0 weeks
Db/Db N=30 Chow Diet 6 weeks old
Db/Db N=30 40% Stearic Diet 6 weeks old
Db/Db N=30 40% Oleic Diet 6 weeks old
2 weeks euthanize 5 mice each group
2 weeks euthanize 5 mice each group
C57KS N=25 Chow Diet 8 weeks old
C57KS N=25 40% Stearic Diet 8 weeks old
C57KS N=25 40% Oleic Diet 8 weeks old
2 weeks
Db/Db N=25 Chow Diet 8 weeks old
Db/Db N=25 40% Stearic Diet 8 weeks old
Db/Db N=25 40% Oleic Diet 8 weeks old
2 weeks euthanize 5 mice each group
2 weeks euthanize 5 mice each group
C57KS N=20 Chow Diet 10 weeks old
C57KS N=20 40% Stearic Diet 10 weeks old
C57KS N=20 40% Oleic Diet 10 weeks old
4 weeks
Db/Db N=20 Chow Diet 10 weeks old
Db/Db N=20 40% Stearic Diet 10 weeks old
Db/Db N=20 40% Oleic Diet 10 weeks old
2 weeks euthanize 5 mice each group
2 weeks euthanize 5 mice each group
C57KS N=15 Chow Diet 12 weeks old
C57KS N=15 40% Stearic Diet 12 weeks old
C57KS N=15 40% Oleic Diet 12 weeks old
6 weeks
Db/Db N=15 Chow Diet 12 weeks old
Db/Db N=15 40% Stearic Diet 12 weeks old
Db/Db N=15 40% Oleic Diet 12 weeks old
2 weeks euthanize 5 mice each group
2 weeks euthanize 5 mice each group
C57KS N=10 Chow Diet 14 weeks old
C57KS N=10 40% Stearic Diet 14 weeks old
C57KS N=10 40% Oleic Diet 14 weeks old
8 weeks
Db/Db N=10 Chow Diet 14 weeks old
Db/Db N=10 40% Stearic Diet 14 weeks old
Db/Db N=10 40% Oleic Diet 14 weeks old
2 weeks euthanize 5 mice each group
2 weeks euthanize 5 mice each group
C57KS N=5 Chow Diet 16 weeks old
C57KS N=5 40% Stearic Diet 16 weeks old
C57KS N=5 40% Oleic Diet 16 weeks old
10 weeks
Db/Db N=5 Chow Diet 16 weeks old
Db/Db =5 40% Stearic Diet 16 weeks old
Db/Db N=5 40% Oleic Diet 16 weeks old
END: 14 weeks euthanize remaining mice each group 24 wks all mice 30 weeks old
24 weeks
END: 14 weeks euthanize remaining mice each group 24 wks all mice 30 weeks old

Figure 2.2.1: Experimental design for 40% high fat diet study. A total of 190 mice, 95 wild-type mice and 95 diabetic (db/db) mice were used in these experiments. Mice were acclimated one week prior to the start of experimental diets and measurements. After the acclimation, five mice per genotype were measured for baseline (before diet initiation) control measurements and then euthanized for tissue processing. The remaining mice were assigned a diet group. Thirty mice of each genotype were assigned to the chow group, 30 were placed in the high fat diet enriched in stearic acid group, and the remaining 30 mice were placed in the high fat diet enriched in oleic acid diet group. After starting the diet, five mice per diet group per genotype were used for measurements and then euthanized for tissue collection every two weeks for the duration of the study with the final group euthanized at 24 weeks on diet.

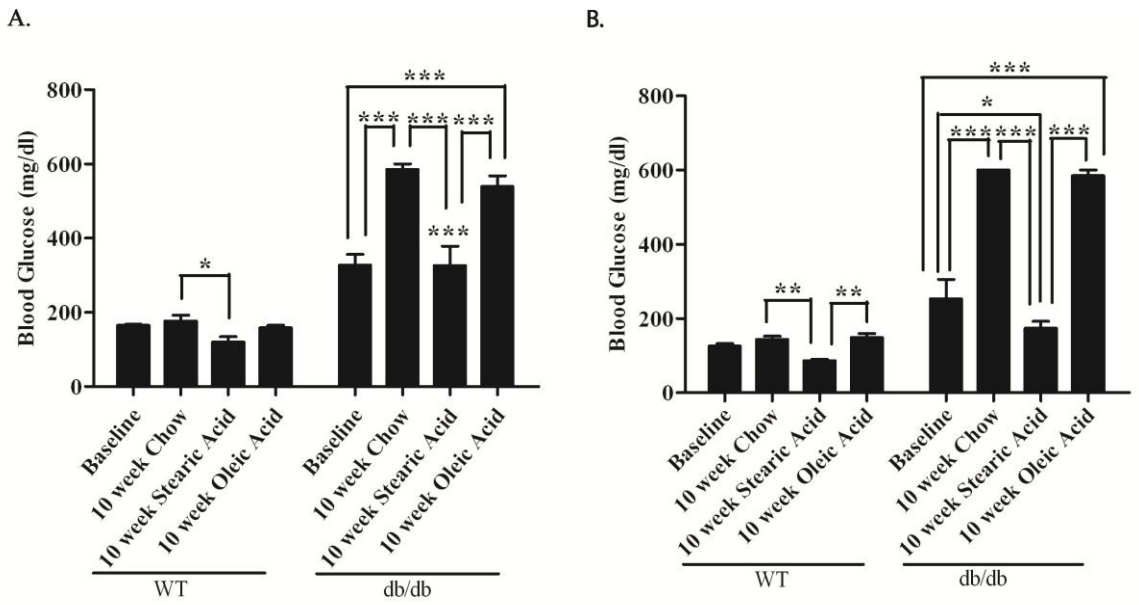


Figure 2.3.1: The high fat diet enriched in stearic acid diet prevents an increase in fasting blood glucose in db/db mice. Five-week-old WT and db/db male mice were fed chow, stearic acid diet or oleic acid diet for 10 weeks. Fasting blood glucose measurements were taken, using commercially available glucometer and tail prick methods. A: Fasting blood glucose levels of mice at baseline (0 weeks on diet) and after 10 weeks on diet. * $p<0.05$, *** $p<0.001$. n = 5 mice per group per genotype. B: Fasting blood glucose levels of mice at baseline and after 10 weeks on diet, second cohort of mice. * $p<0.05$, ** $p<0.01$, *** $p<0.001$. n=5 mice per group per genotype.

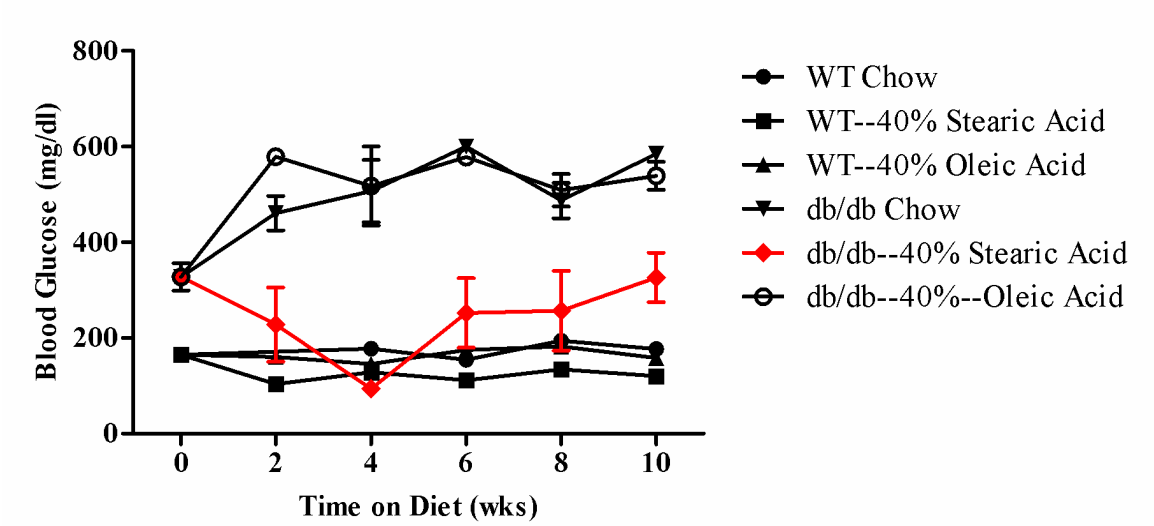


Figure 2.3.2: db/db mice fed a high fat diet enriched in stearic acid had blood glucose levels similar to WT mice after 2 weeks on diet. db/db mice fed a high fat diet enriched in stearic acid had lower blood glucose after 2 weeks of diet administration (red tracing) than the db/db mice fed a high fat diet enriched in oleic acid and normal chow diet. Stearic acid fed db/db mice had blood glucose levels only slightly higher than any of the WT mice. N=5 mice per diet group per time point.

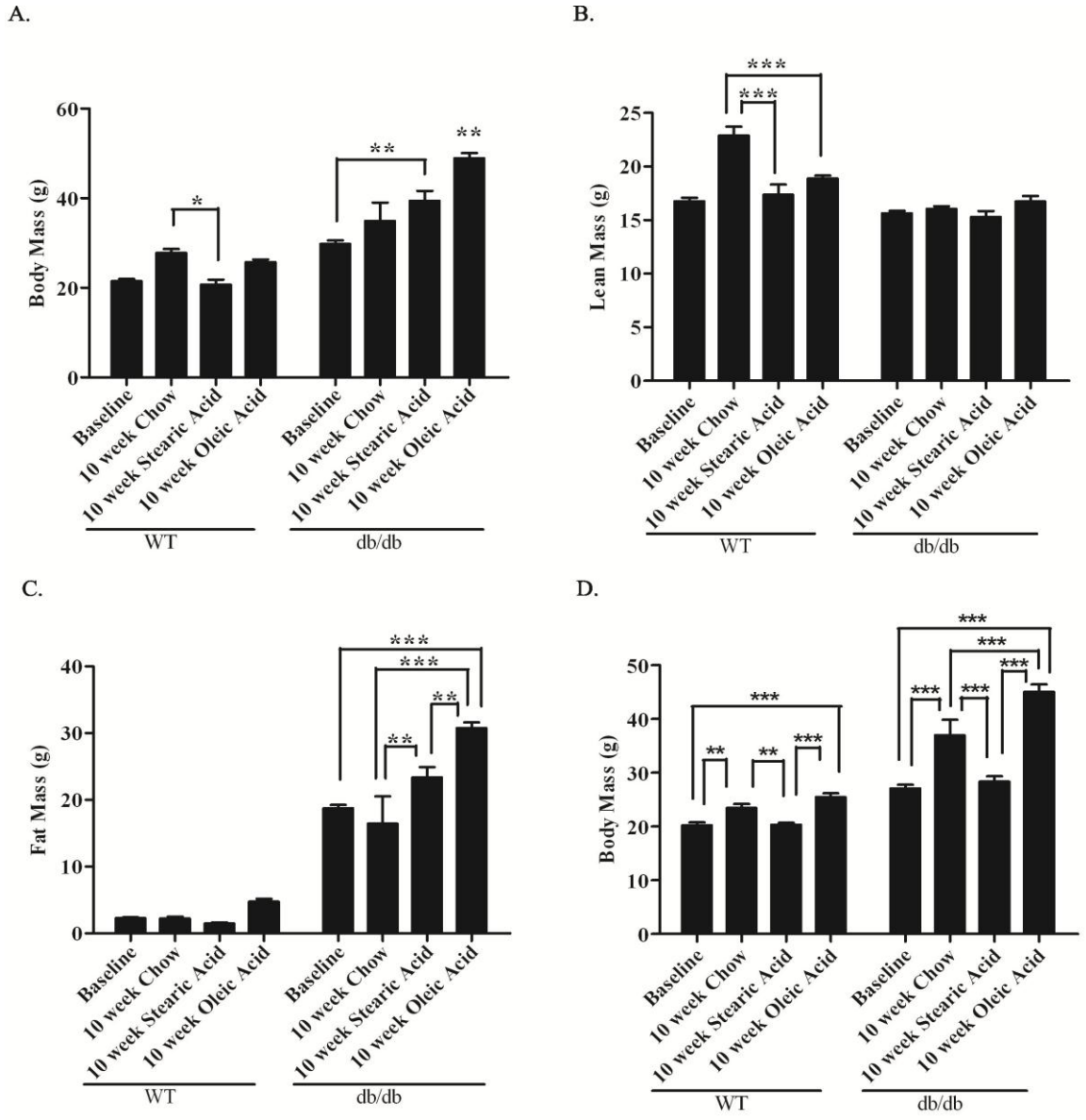


Figure 2.3.3: db/db mice fed a high fat diet enriched in stearic acid do not lose weight. Weight, lean mass, and fat mass were measured using EchoMRI for all mice in the study after ten weeks on diet. A: WT and db/db body mass. db/db 10 week Oleic Acid significantly greater than all other diet groups. B: WT and db/db lean mass. C: WT and db/db fat mass. D: WT and db/db weight after 10 weeks on diet, second cohort of animals. ** $p < 0.01$, *** $p < 0.001$ n= 5 mice per group per genotype.

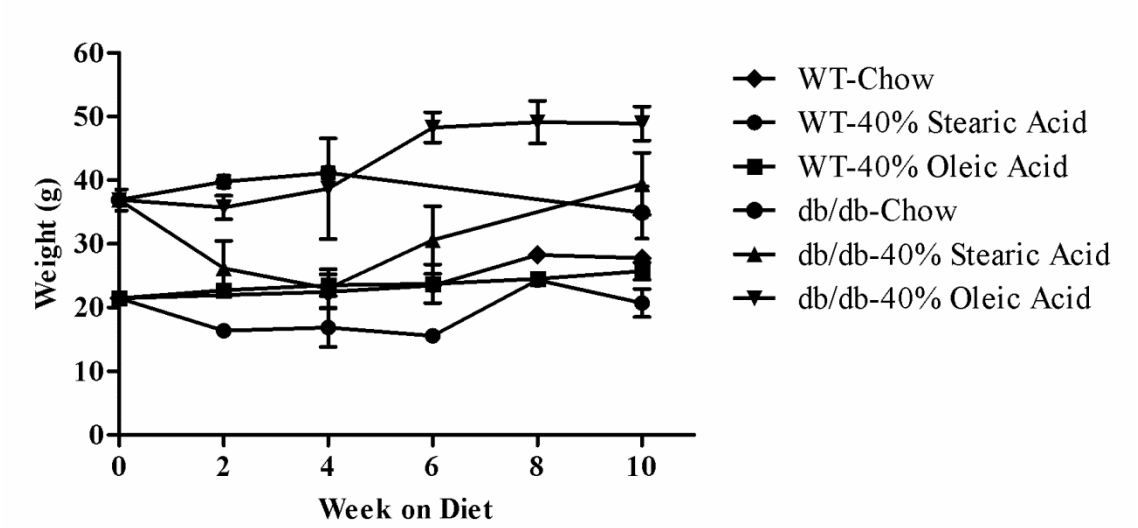


Figure 2.3.4: A diet enriched in stearic acid promoted an early weight loss that was recovered by end of study. Db/db mice fed a high fat diet enriched in stearic acid had an initial decline in weight after 2 weeks on diet. After 4 weeks on diet, the mice gained weight and were no different in weight than their oleic acid or chow fed counterparts by the end of the ten week study. n=5 mice per diet group per time point.

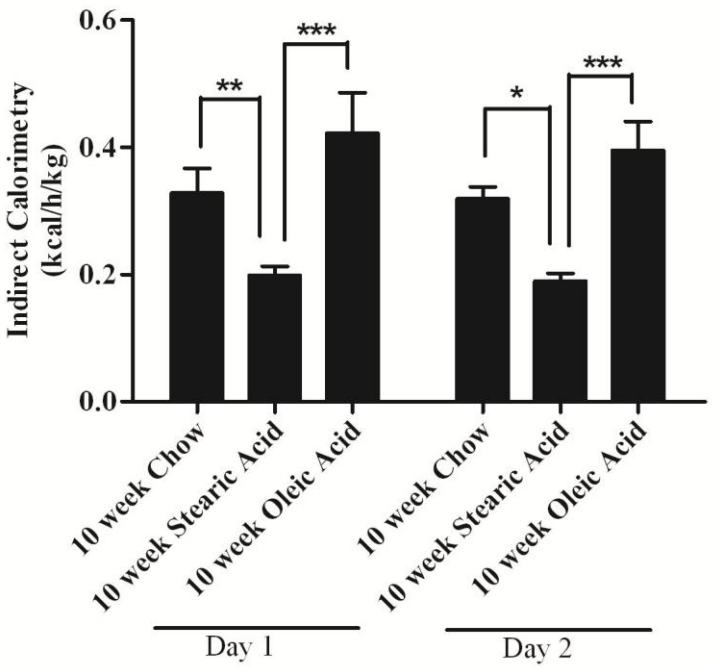


Figure 2.3.5: db/db mice on the high fat diet enriched in stearic acid have lower metabolic rates. Resting metabolic rate was calculated from activity level and oxygen consumption measurements in metabolic monitoring cages. Db/db mice on stearic acid diet had significantly lower resting metabolic rate than db/db mice on chow and oleic acid diets after 10 weeks on diet. * $p<0.05$, ** $p<0.01$, *** $p<0.001$. n=2 mice in chow and oleic acid groups, n=5 mice in stearic acid group.

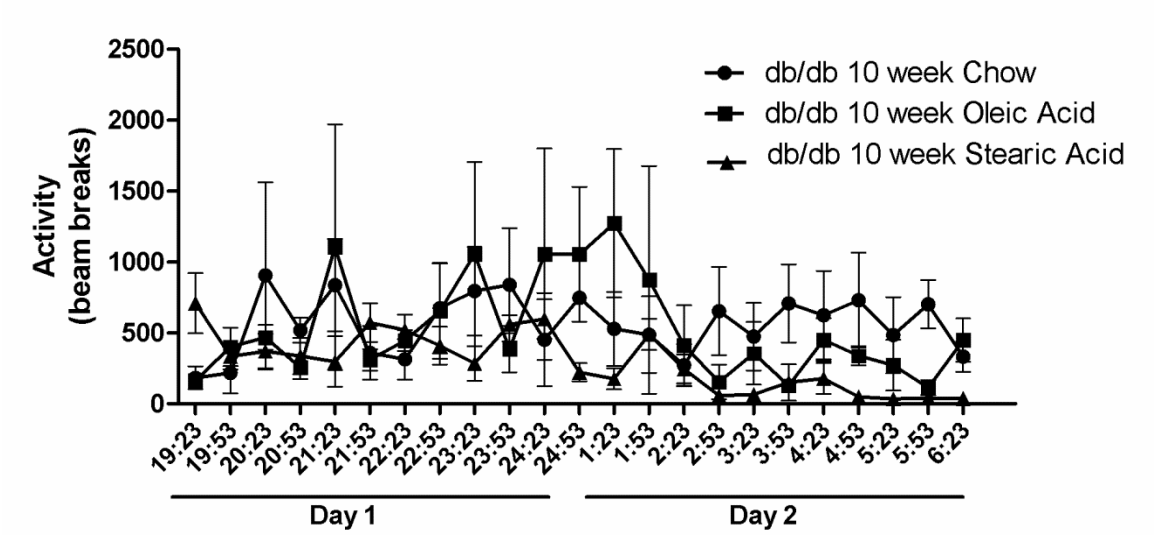


Figure 2.3.6: Stearic acid fed db/db mice showed no difference in activity level as compared to other diet groups. Activity data from calorimetry cages show no differences between diet groups.

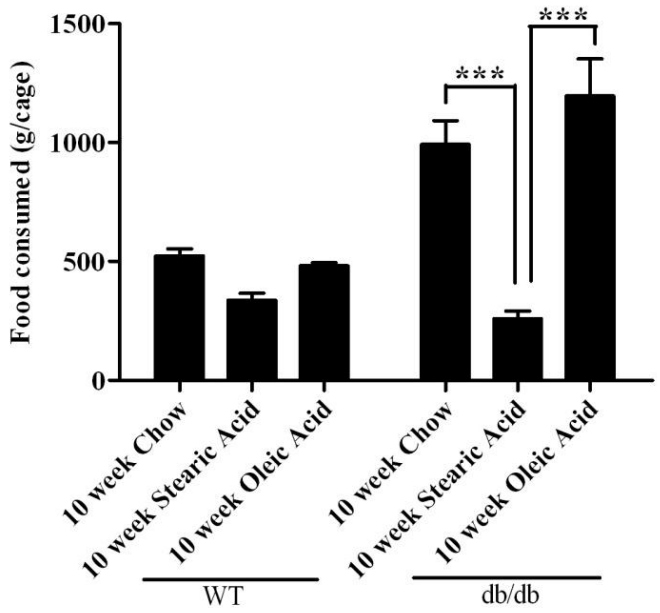


Figure 2.3.7: db/db mice fed a high fat diet enriched in stearic acid consumed less food. Wild-type mice had no significant difference in food consumption over the course of the diet study. Db/db mice fed a high fat diet enriched in stearic acid consumed significantly less food over the course of the ten week diet study as compared to chow or oleic acid fed animals. *** $p<0.001$, n=5 mice per group per genotype.

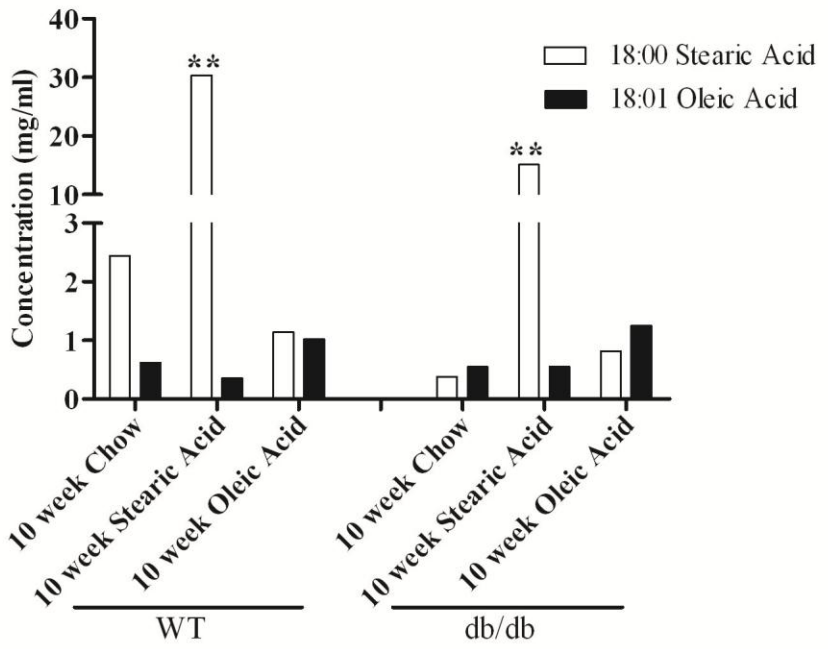


Figure 2.3.8: Dietary stearic acid causes an increase in fecal stearic acid excretion.

Both WT and db/db mice fed a high fat diet enriched in stearic acid had increases in fecal excretion of stearic acid. Feces were collected from the mouse cages. Only the major dietary fatty acids are shown. Fecal fatty acid excretion at 10 weeks on diet from both WT and db/db mice in all three diet groups. ** $p<0.01$ stearic acid compared to all other diet groups within genotype.

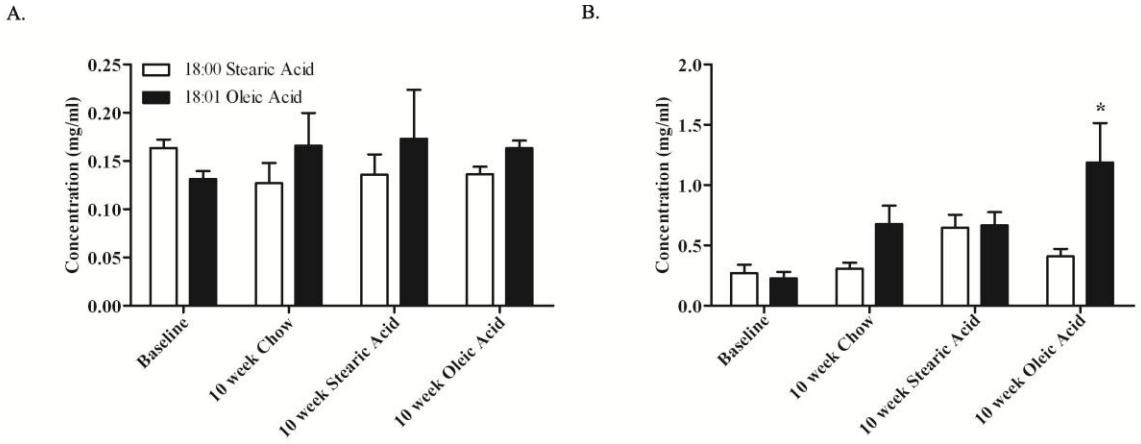


Figure 2.3.9: A high fat diet enriched in stearic acid does not cause accumulation of stearic acid in the liver. Fatty acid amounts in liver determined by GC/MS of livers from WT (A) and db/db mice (B). Note difference in ordinate scale in panel A and B. * $p < 0.05$ for comparison to baseline, n = 5 mice.

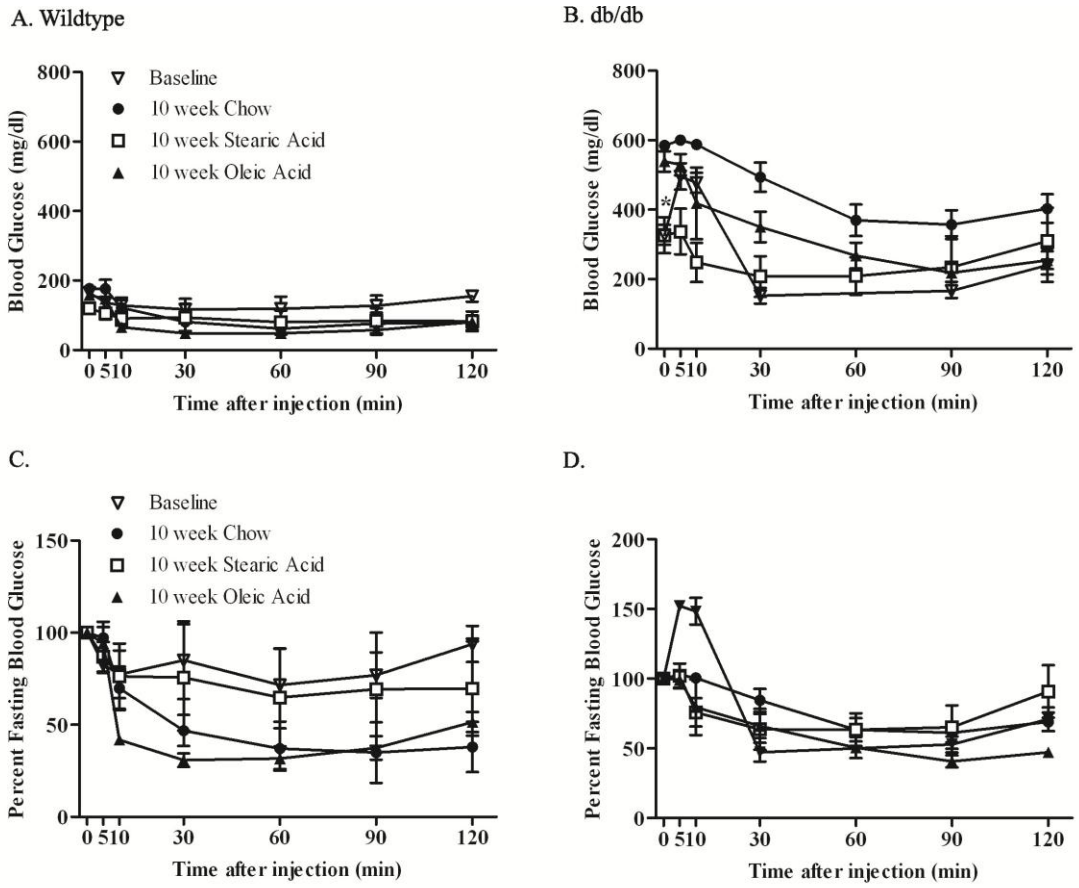


Figure 2.3.10: A stearic acid diet does not alter insulin sensitivity from baseline in db/db mice. Insulin tolerance test was performed on WT (A) and db/db (B) mice. Open triangle represents baseline; filled circle represents chow diet; open square represents stearic acid diet; filled triangle represents oleic acid diet. * $p < 0.05$ db/db mice time 0 stearic vs. time 0 baseline, chow, and oleic diet; n=5 mice. C: WT mice percent fasting blood glucose. D: db/db mice percent fasting blood glucose levels.

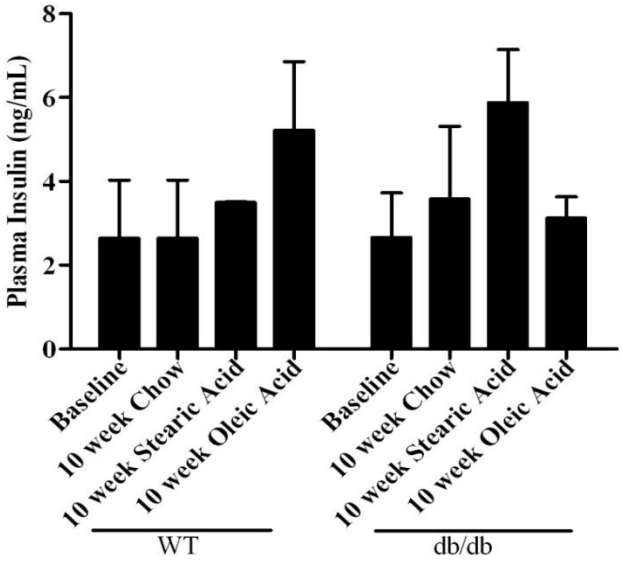


Figure 2.3.11: Dietary stearic acid did not alter plasma insulin concentrations. Plasma insulin concentrations were unchanged from baseline in both WT and db/db mice fed the experimental diets. Though the db/db mice fed the 40% high fat diet enriched in stearic acid tended to have elevated plasma insulin compared to mice fed chow or oleic acid diets, the trend was not significant.

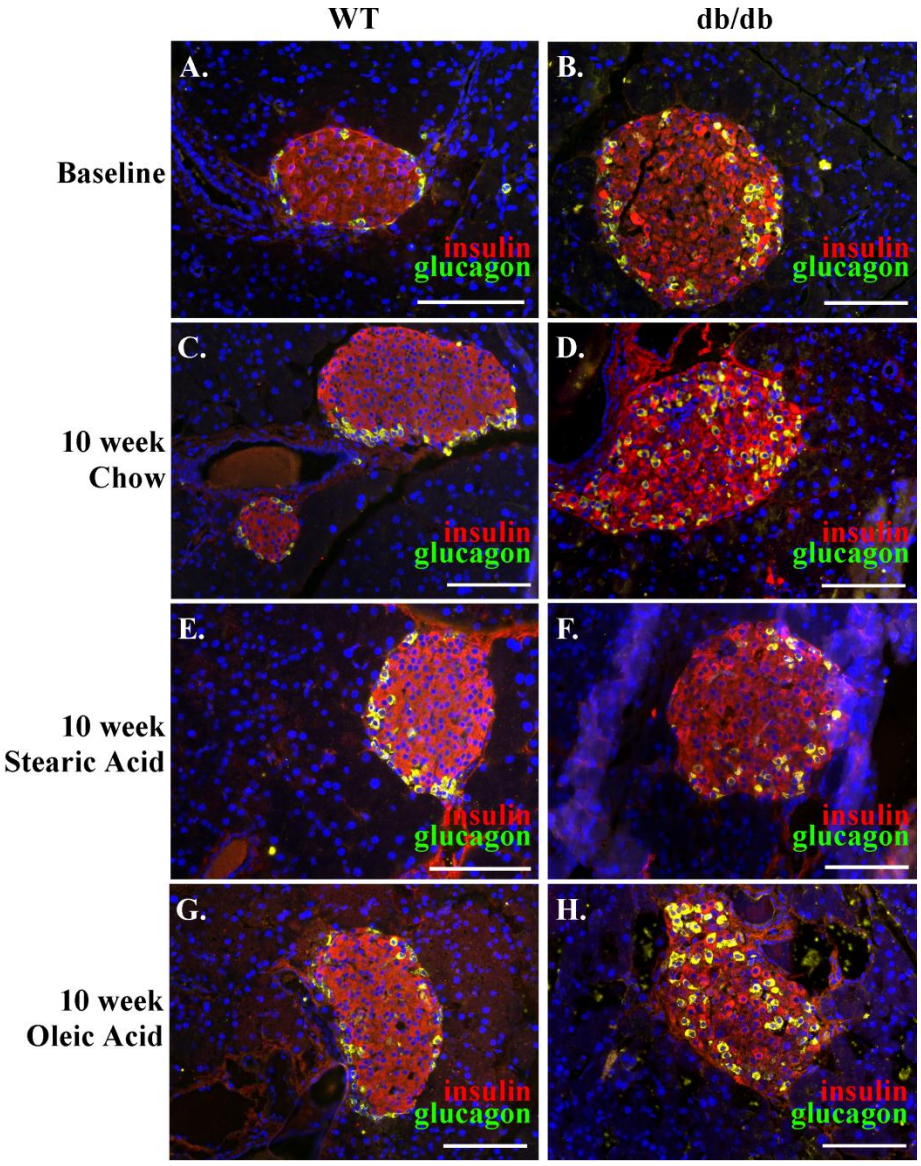


Figure 2.3.12: Stearic acid enriched diet preserves pancreatic islet organization. Representative images of WT and db/db pancreatic islets before and after diet intervention. Baseline (**A** and **B**), 10 week chow diet (**C** and **D**), 10 week stearic acid diet (**E** and **F**), and 10 week oleic acid diet (**G** and **H**). Scale bar is 100µm.

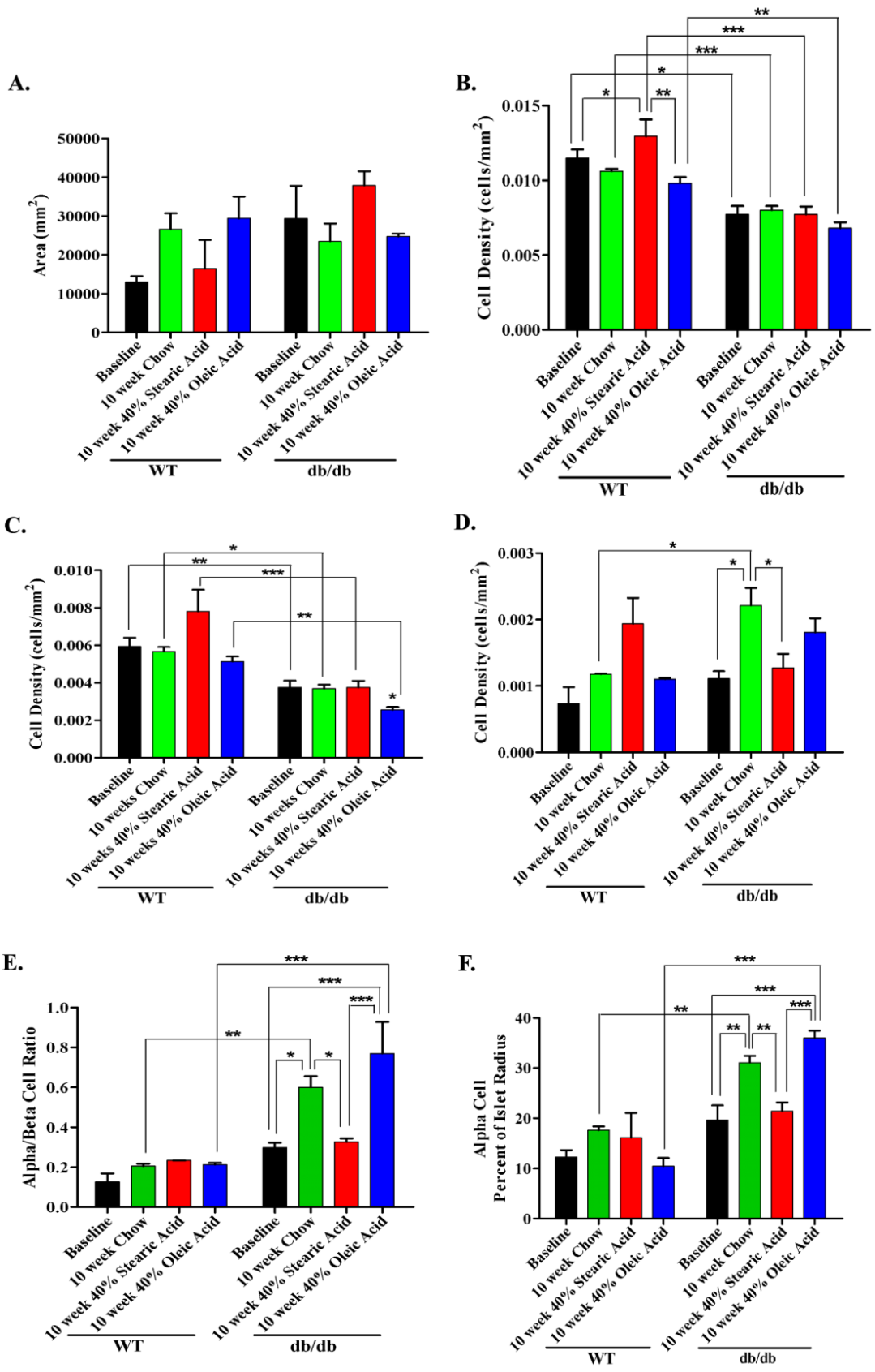

A.
Area (mm2)
50000
40000
30000
20000
10000
0
Baseline
10 week Chow
10 week 40% Stearic Acid
10 week 40% Oleic Acid
WT
db/db
B.
Cell Density (cells/mm2)
0.015
0.010
0.005
0.000
C.
Cell Density (cells/mm2)
0.010
0.008
0.006
0.004
0.002
0.000
10 weeks Chow
10 weeks 40% Stearic Acid
10 weeks 40% Oleic Acid
D.
Cell Density (cells/mm2)
0.003
0.002
0.001
0.000
E.
Alpha/Beta Cell Ratio
1.0
0.8
0.6
0.4
0.2
0.0
F.
Alpha Cell
Percent of Islet Radius
40
30
20
10
0

Figure 2.3.13: A diet enriched in stearic acid preserves pancreatic islet area, total cell density, beta cell density, alpha cell density, and maintains beta cell mass. WT and db/db quantifications of pancreatic islet histology. Total islet area (**A**). Total cell density in islets (**B**). Beta cell density (**C**). Alpha cell density (**D**). Alpha cell to beta cell ratio in islets (**E**). Location of alpha cells in islet as measured by percent of the islet radius (**F**). *p<0.5, ** p<0.01, *** p<0.001.

Chapter 3: Stearic acid effects on the progression of type 2 diabetes in a moderate fat diet in the diabetic mouse model.

3.1 Introduction

High fat diets are linked to diseases such as cardiovascular disease and type 2 diabetes; and, lowering the fat content of the diet offers beneficial results in slowing disease progression. Most Western diets contain at least 36% fat; however, the USDA currently recommends dietary fat content to be much lower, about 20% of the daily intake. Therefore, a moderate fat diet (17% kcal) may be a viable and attractive dietary option for the treatment of type 2 diabetes and more closely align with the current USDA and AHA recommendations than the 40% high fat diets. Therefore, decreasing the overall fat content in the diet will be more fitting to these current dietary guidelines.

Encouraged by the positive results from the 40% kcal stearic acid enriched high fat diet study, I hypothesized that a moderate fat diet (17% kcal from fat) enriched in stearic acid may offer the same protection against the progression of type 2 diabetes while more closely aligning with the current dietary recommendations. Wild-type and diabetic mice were fed moderate fat diets for ten weeks and were assessed for progression of type 2 diabetes during the study.

Diabetic mice fed a moderate fat diet (17% fat) enriched in stearic acid were not protected from the progression of type 2 diabetes; however, the db/db mice fed the stearic acid enriched diet had blood glucose levels that were lower than the db/db mice fed the chow diet. Interestingly, all mice, both wild-type and db/db, had increased accumulation of oleic acid in the liver regardless of experimental diet.

3.2. Methods and Materials

3.2.1 Animals

Age-matched, male C57BLKS/J (WT, n=105) and BKS.Cg-*Dock7*^{m} +/+ *Lepr*^{db}/J (db/db, n=105) mice were purchased from The Jackson Laboratory (Bar Harbor, Maine) at four- five weeks of age. All mice were allowed to acclimate for one week and fed *ad libitum* a commercially available rodent chow diet (Teklad Global 18% Protein Rodent Diet TD.2018) obtained from Harlan Laboratories (Madison, Wisconsin). Mice were kept on a 12-hr light/dark cycle at 25°C throughout the study. At five weeks of age, all mice were weighed, ear tagged, and randomly assigned to a study group. The three study groups were fed different diets: chow diet or one of two experimental diets (17% kcal stearic acid diet, Harlan Teklad TD.03459, 17% kcal oleic acid diet TD.09315). All mice were maintained one of these diets *ad libitum* for 10 weeks. Every two weeks for the duration of the study, metabolic measurements were performed using five mice per diet group per genotype. Weight, blood glucose and insulin tolerance measurements were taken at the start of the diet and at the endpoint in this cohort of animals. **Figure 3.2.1** shows the experimental design for this study. Animal care and housing conducted according to the NIH *Guide for the Care and Use of Laboratory* Animals. All experiments were approved by Institutional Animal Care and Use Committee at the University of Kentucky Animal Housing Facility and overseen by University of Kentucky veterinarians.

3.2.2 Diets

Table 3.2.1 and **Table 3.2.2** list the nutritional value and composition of each diet used in this study. The stearic and oleic acid diets used in this study contained similar

percentages of protein and carbohydrate whereas chow diet contained slightly more of total kcal from protein and 60% total kcal from carbohydrates. The fat content of each diet was unique. The experimental diets were custom made by Harlan Teklad using a modified TD.03459 diet. A Harlan Teklad nutritionist calculated all dietary nutritional values.

3.2.3 Glucose Measurements and Insulin Tolerance Test

Insulin tolerance tests were performed every two weeks during the study on five mice per diet group per genotype. Mice were weighed and then fasted for four hours in a clean cage prior to testing. Fasting blood glucose was measured by tail prick with a commercially available glucometer and test strips (One Touch Ultra Glucose Monitoring Kit, Lifescan, Milpitas, California). Mice were injected (i.p.) with insulin (2mU/g) and blood glucose was measured (mg/dl) 0, 5, 10, 30, 60, 90 and 120 minutes after insulin injection. If blood glucose fell below 20mg/dL, mice were rescued with 200uL of 20% glucose solution (i.p.) and excluded from the ITT experiment. Blood glucose measurements were normalized to baseline measurements, and both sets of data plotted over time.

3.2.4 Insulin ELISA

Every two weeks, collected blood from fasted animals (4 hour fast) was allowed to clot for 20 minutes in a vacutainer and centrifuged at 1500 X g for 10 minutes to isolate serum. Serum was then snap-frozen and stored at -80^{0}C until analyzed. Plasma insulin levels were measured using a commercially available mouse/rat insulin ELISA (Millipore, Billerica, Massachusetts) and reported in ng/mL.

3.2.5 Body Composition

Every two weeks during the study, mouse body composition, including fat mass and lean mass, was determined using EchoMRI Quantitative Magnetic Resonance Body Composition Analyzer (Echo Medical Systems, Houston, Texas) on conscious mice (n=5 per diet per genotype). Conscious mice were placed into the measuring tube and the tube placed into the EchoMRI machine. The EchoMRI uses the distinctions in NMR amplitude signals of various tissues to determine mass of muscle, fat and body fluids.

3.2.6 Oxygen Consumption

Every two weeks during the study, five mice per diet group per genotype were placed in oxygen consumption chambers for indirect calorimetry measurement. Metabolic rate was indirectly determined using weight, temperature and oxygen consumption. Conscious mice were weighed and placed in oxygen consumption chambers. Room air was pumped into and out of the chambers and analyzed for oxygen and carbon dioxide content (mL/hr) using a CWE metabolic monitoring system and software (CWE, Allentown, Pennsylvania). Oxygen consumption was then computed from these measurements using the equation VO2/weight (mL/g/hr).

3.2.7 Gas Chromatography/Mass Spectrometry

Tissue, serum, and feces were collected post-mortem every two weeks during study. Total fatty acids were extracted from 100mg of samples using Folch method. Extractions were then analyzed using GC/MS. Briefly, a 1 µl sample was injected into the gas chromatography system (model 6890GC G2579A system; Agilent, Palo Alto, California) equipped with a column (J&W DB5HT capillary columns, Agilent Technologies) and a flame ionization detector. An Agilent 5973 network mass selective

detector was used to identify target peaks of individual fatty acids. First, the corrected peak area of the FA of interest in the sample was calculated by multiplying by the ratio of peak area of the internal standard in standard set and the peak area of the internal standard in the sample and then multiplying by the ratio of the internal standard concentration in standard set and sample. Second, the concentration of the FA of interest was calculated by multiplying the corrected area of the interest peak by the ratio of the interest FA in the standard and in the sample. The two-step formula used is below.

$$\mathrm{A_{c,x}} = \mathrm{A_x} * \left(\frac{\mathrm{A_{C17,standard}}}{\mathrm{A_{C17,sample}}}\right) * \left(\frac{\mathrm{CONC_{C17,sample}}}{\mathrm{CONC_{standard}}}\right)$$

$\mathrm{A_x}$ denotes the peak area of the fatty acid of interest in the sample. The area of the peak is corrected for sample variation and $\mathrm{A_{c,x}}$ denotes the corrected peak area of the fatty acid of interest in the sample. $\mathrm{A_{C17,standard}}$ and $\mathrm{A_{C17,sample}}$ represent the area of the internal standard in the standard solution and in the sample. Next, the sample concentration was calculated.

$$CONC_x = A_{c,x} * \left(\frac{CONC_{x,standard}}{A_{x,standard}}\right)$$

3.2.8 Data Analysis

Values are depicted as mean $\pm$ standard error and considered significant if $p < 0.05$. Data were statistically analyzed using two-way ANOVA with Bonferroni correction or one-way ANOVA with Dunnett comparison posttest when appropriate using GraphPad Prism 5.01 for Windows (GraphPad Software, San Diego, California). NIS-Elements 3.0 (Nikon Instruments, Elgin, Illinois) was used for islet area and staining analysis.

3.3 Results

3.3.1 After 10 weeks on moderate fat diet, db/db mice fed stearic acid had blood glucose levels lower than chow fed mice.

Fasting blood glucose levels were determined at the beginning of the moderate fat study (Baseline) and every two weeks during the ten week study for both WT and db/db mice (two week data was not collected due to fire alarm and mandatory evacuation of the building). These data shown in **Figure 3.3.1** are for baseline and measurements taken after 10 weeks on diet. For WT mice on chow, stearic acid, or oleic acid diet the blood glucose levels did not change throughout the study. The baseline glucose levels for db/db mice were about 1.5-fold higher than WT mice, as expected (38). Db/db mice fed chow and oleic acid diets approximately doubled their blood glucose levels in ten weeks. In contrast, db/db mice fed stearic acid had increased blood glucose levels over baseline, but the level at ten weeks was significantly lower than the chow fed db/db mice.

3.3.2 Weight and body composition remained unchanged after moderate fat feeding.

In diabetic models, lowered blood glucose levels are usually linked to decreases in weight (41, 59, 83). The db/db mice placed on moderate fat diets had no changes in weight after ten weeks on diet (**Figure 3.3.2A**). The db/db mice had no changes in fat mass vs. baseline after ten weeks on diet (**Figure 3.3.2B**). Interestingly, the db/db mice fed stearic acid had decreased lean mass from baseline and also compared to chow and oleic acid fed db/db mice (**Figure 3.3.2C**). WT mice gained weight over the course of the diet study with oleic and stearic acid fed mice gaining more weight than chow fed mice (**Figure 3.3.2A**).WT mice fed oleic diet gained fat mass from baseline and more than mice fed chow (**Figure 3.3.2B**). WT mice fed chow diet gained lean mass during the

study (**Figure 3.3.2C**). These results contradict the current dietary recommendations that all people consume a low to moderate fat diet. Even though the WT mice had no change in blood glucose levels, the increase in weight may predict increased frequency of health problems in older individuals.

3.3.3 Oxygen consumption was unchanged by moderate fat diet enriched in stearic acid.

Increases in resting metabolic rate or activity could result in lower blood glucose levels without concomitant weight loss. To assess these alternate explanations, I measured oxygen consumption as an indirect measure of metabolic rate of the mice after 10 weeks on diet (**Figure 3.3.3**). All db/db mice had similar oxygen consumption measurements. The moderate fat diet enriched in stearic acid had no effect on oxygen consumption and, by extension, no effect on metabolic rate. Therefore, the lowered blood glucose measurements observed in the db/db mice fed the stearic versus oleic lower fat diet cannot be explained by alterations in metabolic rate.

3.3.4 Fatty acids, especially oleic acid, accumulate in the livers of WT and db/db mice fed 17% kcal enriched in stearic acid and oleic acid.

The liver is a major site of fatty acid accumulation in diabetic mice. **Figure 3.3.4** illustrates that the db/db mice fed the moderate fat diets containing either stearic acid or oleic acid had greater accumulation of fatty acid in the liver than wild-type mice (*$p<0.05$, n=5 per diet group per genotype), as previously documented for db/db mice (147). Surprisingly, both the oleic and stearic acid enriched diets, but not the chow diet, caused fatty acid accumulation in the liver of db/db mice. The increased fatty acid

accumulation in the liver, especially in the oleic acid fed db/db mice, is contradictory to the current opinion that an unsaturated fat diet is beneficial to the diabetic condition.

3.3.5 After feeding a moderate fat diet, insulin tolerance is unchanged in WT and db/db mice.

A reduction of fat absorption and lower blood glucose may also indicate improved insulin tolerance. High fat feeding has shown to exacerbate insulin resistance in the diabetic mouse model (7, 13, 14, 16, 28, 30). Diets rich in unsaturated fat, especially oleic acid, and low fat diets decrease insulin resistance (16, 136, 154, 163, 170). Surprisingly, WT and diabetic mice had no change from baseline in insulin response after ten weeks on diet (**Figure 3.3.5 A** and **B)**. When plotted as a percent of fasting blood glucose measurement, WT mice had no difference in blood glucose measurements. However, the db/db mice fed oleic acid diet had higher blood glucose measurements after 10 minutes post insulin injection (**Figure 3.3.5 C** and **D**).

3.3.6. Plasma insulin content was similar in all diet groups.

Following the same course as insulin sensitivity, plasma insulin levels were also measured after 10 weeks on diet. If insulin sensitivity was unchanged, I expected that plasma insulin content would also be unchanged. Indeed, I found there were no differences among diet groups (**Figure 3.3.6**). This is a surprising result because moderate fat diets are the benchmark for improving insulin sensitivity thereby also lowering plasma insulin levels.

3.4 Discussion

In this study, I investigated if a moderate fat diet enriched in stearic acid would provide protective benefits against the progression of type 2 diabetes. I found that mice on a moderate fat diet (17% kcal from fat) enriched in stearic acid, a saturated fat, had lower blood glucose levels than the standard chow fed db/db mice after ten weeks of feeding. Over the course of the diet study, db/db mice fed stearic acid enriched diet had increased blood glucose levels as compared to the baseline measurements, but when compared to chow and oleic fed mice, the db/db mice fed stearic acid had lower blood glucose levels. Normally, a decrease in weight and/or an increase in metabolic rate are linked to lower blood glucose levels. I found that after 10 weeks of moderate fat diet, weight and oxygen consumption did not change. Therefore, there was no concomitant change in weight or metabolic rate to account for the lower blood glucose levels in the db/db mice fed a moderate fat diet enriched in stearic acid. Interestingly, all mice, both wild-type and db/db, fed the experimental diets had increased accumulation of fatty acids in the liver. Since the mice fed the moderate fat diets all had similar body composition and oxygen consumption, the lower blood glucose level observed in the db/db mice fed the stearic acid diet may have been caused by a defect in absorption as I have previously reported with a 40% high fat diet enriched in stearic acid.

The absorption of stearic acid in previous studies has depended on the form of the fat, either liquid or solid (10, 22, 75, 90). Diets enriched in a liquid form of stearic acid did not result in lowered fat absorption. However, the diets that were enriched in solid stearic acid resulted in decreased fat absorption (10). In this study, I used stearic acid in its natural, solid form, which would be the easiest form of stearic acid to incorporate into

a diet. This solid form also ensures that the fat is a pure form of stearic acid and is not contaminated with other fatty acids that may alter or influence our results.

Most of the studies that involve stearic acid have been cardiovascular studies, but a few have evaluated the effects of saturated fats on type 2 diabetes. Berry, et. al. showed that mono-unsaturated fats have positive effects on insulin sensitivity in type 2 diabetes as long as saturated fats are kept at a minimum (16). In this study, I have shown that even a moderate fat diet enriched in oleic acid, a mono-unsaturated fat, promotes obesity and has no effect on insulin sensitivity. The oleic acid diet presented here closely aligns with the current USDA and AHA dietary recommendations of increasing mono-unsaturated fats, low saturated fats and decreased overall fat content in the diet (31).

Oleic acid has been heralded in cardiovascular research for its ability to lower plasma cholesterol levels (25, 27). The mice fed oleic acid in this study had increased oleic acid accumulation in the liver. While liver fatty acid accumulation is not a direct measure of plasma lipoprotein content, it is a good indicator of plasma content since the liver is the storage depot for fatty acids and secretes lipoproteins (51). Accumulations of fatty acids in the liver have been linked to insulin resistance. In this study, I found that db/db mice fed a diet enriched in oleic acid did not lower blood glucose levels nor did it reverse insulin tolerance. Previous studies have reported diets rich in the unsaturated fat, oleic acid, have been shown to be beneficial in lowering blood glucose levels and reversing insulin intolerance in diabetic patients (16, 29, 50, 89, 90, 104, 124, 125, 154). This study indicates that, at least in db/db mice, oleic acid does not have the beneficial effects previous reported when administered in a single fat enriched diet. Additionally,

these results indicate that oleic acid may have detrimental effects by accumulating in the liver.

In this study, the saturated fat, stearic acid, had beneficial effects on blood glucose levels and did not accumulate in the liver. These results support the argument that all dietary fats are not the same and stearic acid, a saturated fat, has beneficial effects. Cardiovascular studies have shown that stearic acid does not promote increases in plasma cholesterol levels. Whether or not this is due to defects in absorption of stearic acid is still unknown. However, the overall effect of stearic acid has been promising in this study as a potential dietary modification for treating type 2 diabetes.

Limiting dietary fat content to less than 20% of the total kilocalories did not prevent an increase in blood glucose levels, a marker for the progression of type 2 diabetes. The stearic acid fed db/db mice did not have a lower body mass than the baseline or chow fed diet group, but did have a decrease in lean mass. This decrease in lean mass may indicate that the mice were not eating enough food and/or were using breaking down muscle to maintain body weight. This is contradictory to the wild-type mice fed the stearic acid diet which increased lean mass from baseline. The oleic acid mice were not significantly greater in body mass at the end of the diet study than the chow group or the baseline measurement. Additionally, WT mice fed the experimental diets gained weight over the course of the diet revealing that a moderate fat diet enriched in one type of fat is not useful tool to halt the progression of type 2 diabetes in the db/db mouse model.

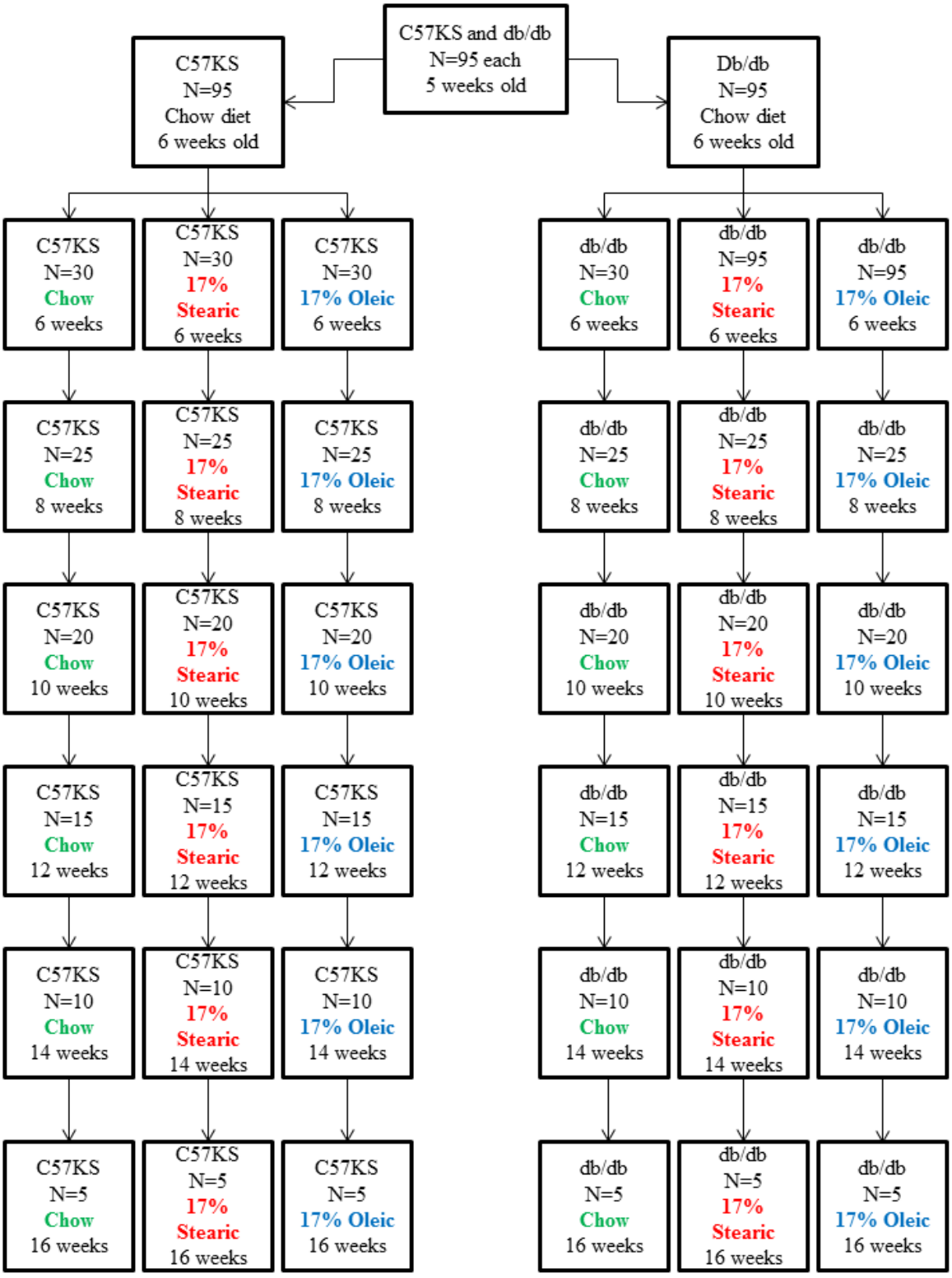

C57KS and db/db
N=95 each
5 weeks old
C57KS
N=95
Chow diet
6 weeks old
Db/db
N=95
Chow diet
6 weeks old
C57KS
N=30
Chow
6 weeks
C57KS
N=30
17%
Stearic
6 weeks
C57KS
N=30
17% Oleic
6 weeks
db/db
N=30
Chow
6 weeks
db/db
N=95
17%
Stearic
6 weeks
db/db
N=95
17% Oleic
6 weeks
C57KS
N=25
Chow
8 weeks
C57KS
N=25
17%
Stearic
8 weeks
C57KS
N=25
17% Oleic
8 weeks
db/db
N=25
Chow
8 weeks
db/db
N=25
17%
Stearic
8 weeks
db/db
N=25
17% Oleic
8 weeks
C57KS
N=20
Chow
10 weeks
C57KS
N=20
17%
Stearic
10 weeks
C57KS
N=20
17% Oleic
10 weeks
db/db
N=20
Chow
10 weeks
db/db
N=20
17%
Stearic
10 weeks
db/db
N=20
17% Oleic
10 weeks
C57KS
N=15
Chow
12 weeks
C57KS
N=15
17%
Stearic
12 weeks
C57KS
N=15
17% Oleic
12 weeks
db/db
N=15
Chow
12 weeks
db/db
N=15
17%
Stearic
12 weeks
db/db
N=15
17% Oleic
12 weeks
C57KS
N=10
Chow
14 weeks
C57KS
N=10
17%
Stearic
14 weeks
C57KS
N=10
17% Oleic
14 weeks
db/db
N=10
Chow
14 weeks
db/db
N=10
17%
Stearic
14 weeks
db/db
N=10
17% Oleic
14 weeks
C57KS
N=5
Chow
16 weeks
C57KS
N=5
17%
Stearic
16 weeks
C57KS
N=5
17% Oleic
16 weeks
db/db
N=5
Chow
16 weeks
db/db
N=5
17%
Stearic
16 weeks
db/db
N=5
17% Oleic
16 weeks

Figure 3.2.1: Experimental design for 17% moderate fat diet study. A total of 190 mice, 95 wild-type mice and 95 diabetic (db/db) mice were used in these experiments. Mice were acclimated one week prior to the start of experimental diets and measurements. After the acclimation, five mice per genotype were measured for baseline (before diet initiation) control measurements and then euthanized for tissue processing. The remaining mice were assigned a diet group. Thirty mice of each genotype were assigned to the chow group, 30 were placed in the high fat diet enriched in stearic acid group, and the remaining 30 mice were placed in the high fat diet enriched in oleic acid diet group. After starting the diet, five mice per diet group per genotype were used for measurements and then euthanized for tissue collection every two weeks for the duration of the study with the final group euthanized at 24 weeks on diet.

Table 3.2.1: Dietary Components

	Catalog Number	Protein	Carbohydrate	Fat
Chow	TD. 2918	18.80	53.80	17.0
17% kcal Stearic Acid	TD. 03459	19.00	64.40	16.60
17% kcal Oleic Acid	TD. 09315	18.80	63.90	17.20

Table 3.2.2: 17% Diet Fat Composition

Diet	Chow	Stearic Acid	Oleic Acid
% kcal from Fat	17.00	16.60	17.20
% Stearic acid in fat	2.60	85.00	2.00
% Oleic acid in fat	22.50	1.00	65.00
% Other Essential Fatty Acids	74.90	14.00	34.00

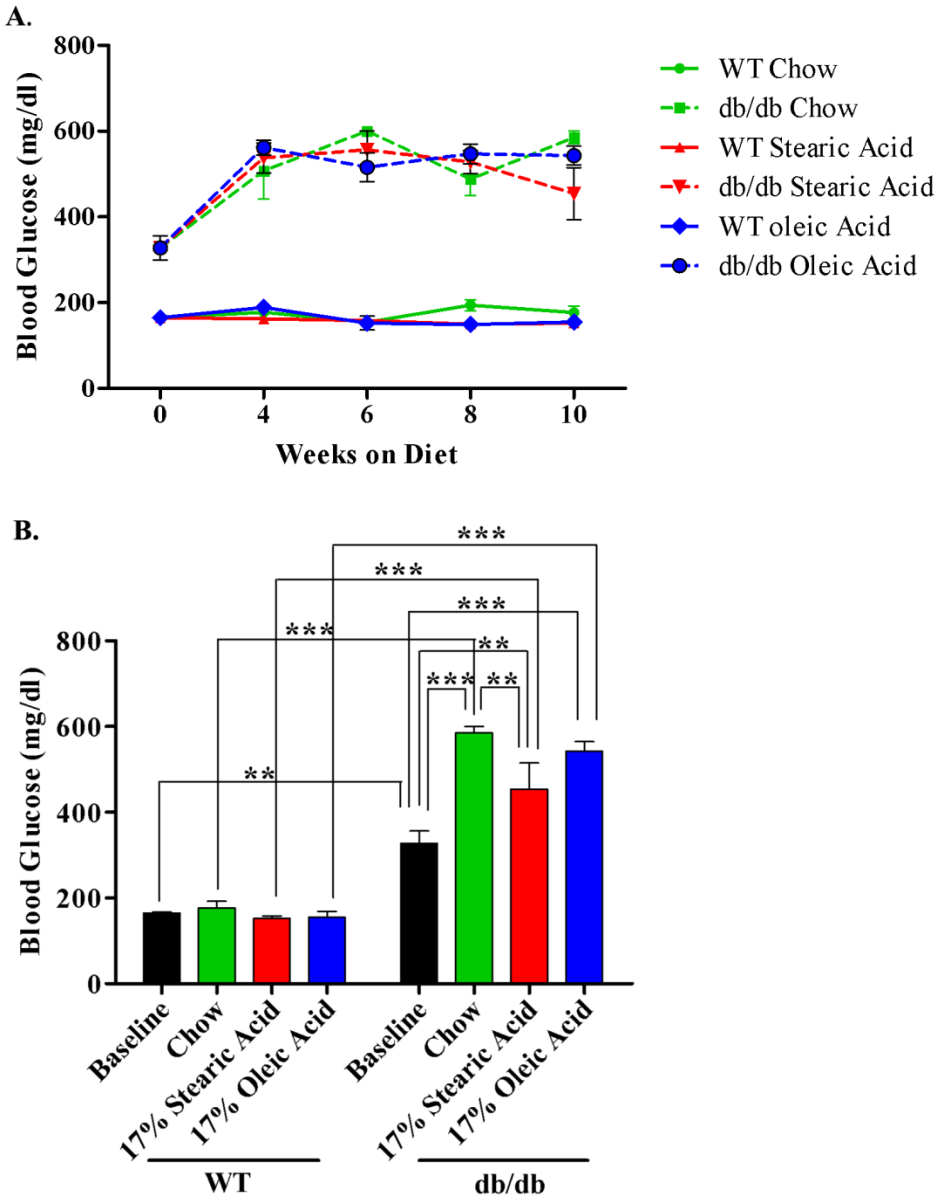


Figure 3.3.1: A moderate fat diet enriched in stearic acid did not prevent an increase in blood glucose levels in diabetic mice. WT and db/db mice were fed a either a moderate fat diet enriched in stearic acid or oleic acid or were fed standard chow diet for 10 weeks. After ten weeks on diet, all diabetic mice experienced an increase in blood glucose levels. Note no two week data is present due to fire alarm. ** $p<0.01$, *** $p<0.0001$; n=5.

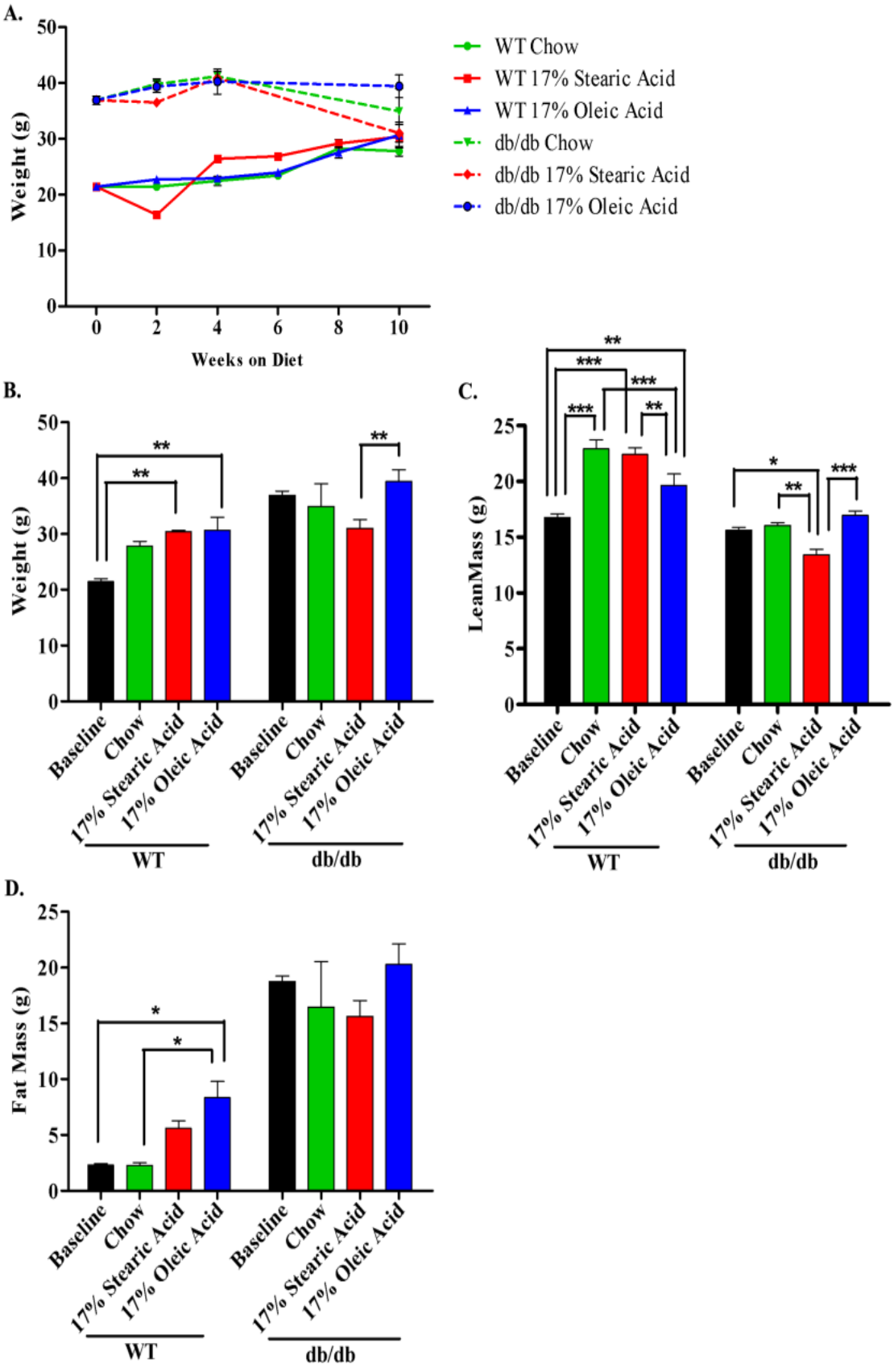

A.
Weight (g)
Weeks on Diet
WT Chow
WT 17% Stearic Acid
WT 17% Oleic Acid
db/db Chow
db/db 17% Stearic Acid
db/db 17% Oleic Acid
B.
Weight (g)
C.
LeanMass (g)
D.
Fat Mass (g)
Baseline
Chow
17% Stearic Acid
17% Oleic Acid
WT
db/db

Figure 3.3.2: A moderate fat diet enriched in stearic acid did not promote weight gain in db/db mice. After 10 weeks on diet, WT mice fed stearic acid and oleic acid diets had increases in weight gain (**A**. **p<0.01, n=5). In oleic acid fed WT mice had increases in fat mass from baseline. WT mice fed oleic acid also had increased fat mass compared to chow fed animals. (**B**. *p<0.05, n=5). All WT mice had increases in lean mass in all diet groups compared to baseline. Chow and stearic acid fed WT mice had higher increase in lean mass compared to oleic acid fed WT mice. Db/db mice fed stearic acid had lost lean mass after 10weeks of diet compared to baseline and chow and oleic acid diet groups (**C**. *p<0.05, **p<0.01, ***p<0.001, n=5).

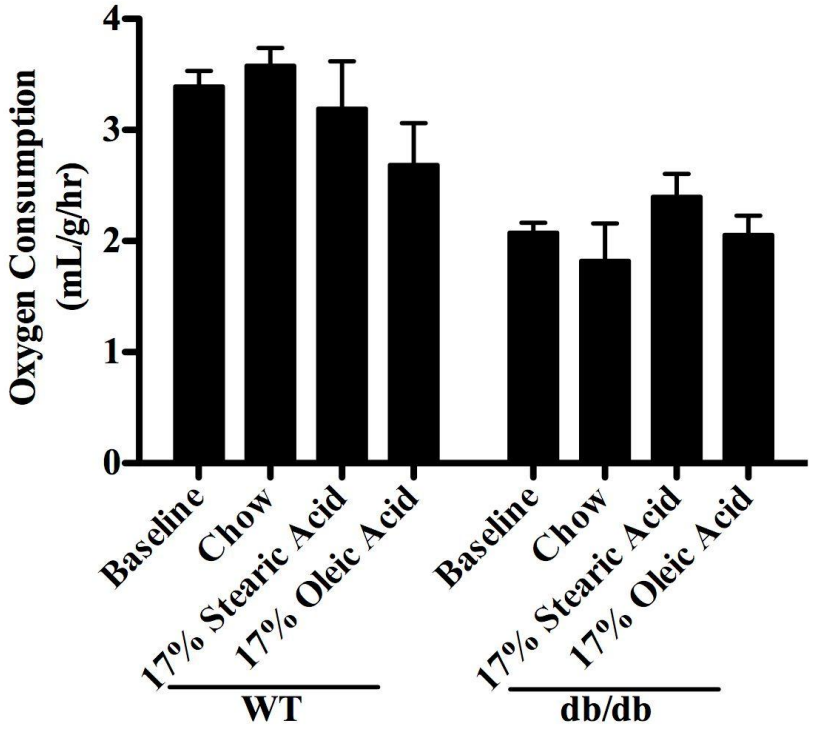


Figure 3.3.3: A moderate fat diet does not alter oxygen consumption. Oxygen consumption was not changed by any diet. WT and db/db mice showed a slight and insignificant variation in oxygen consumption, as expected.

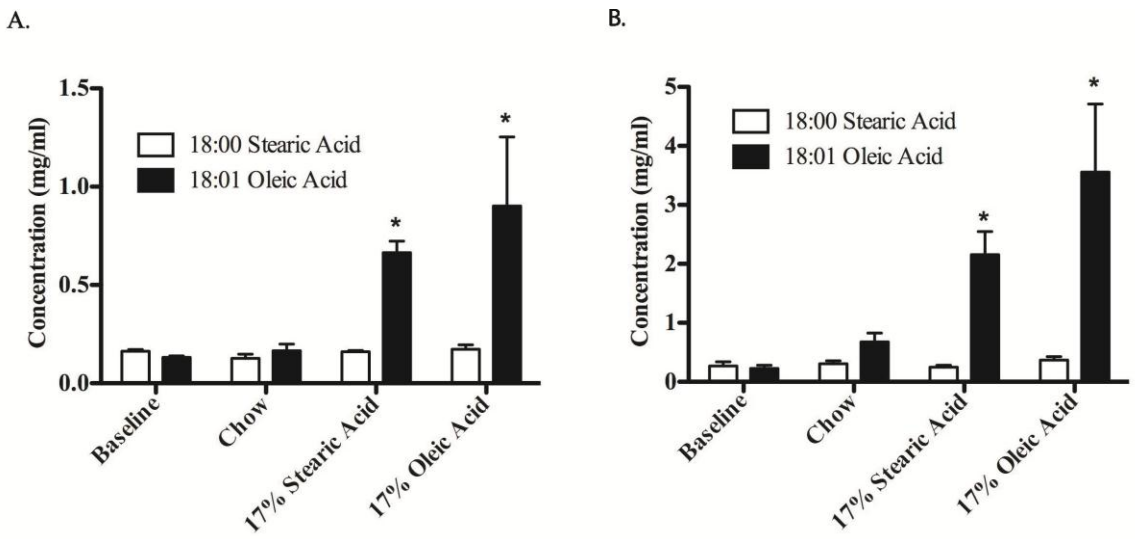


Figure 3.3.4: Liver Fatty Acid Accumulation. Fatty acids accumulated in the livers of both the WT and db/db mice fed the experimental diets. Interestingly, in the livers of both WT and db/db mice, oleic acid was the major fat that accumulated, regardless of diet. Note the difference in ordinate scale in panel A and B. * $p<0.05$, n=5 mice.

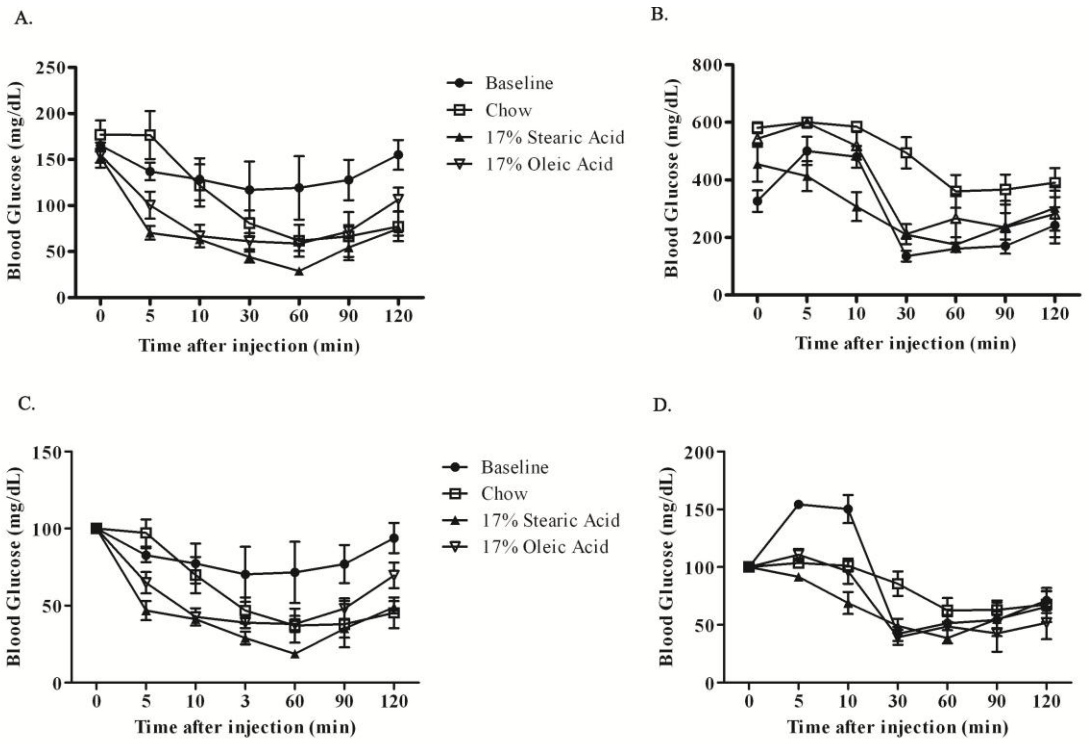


Figure 3.3.5: Insulin Tolerance Test. Insulin tolerance remained unchanged in both WT (**A**) and db/db mice after ten weeks on diet (**B**). When blood glucose was plotted as percent of fasting blood glucose, no differences were observed. Interestingly, in WT mice (**C**), all diet groups had lower percent of baseline blood glucose levels as compared to baseline. Db/db mice also showed a similar trend. Db/db baseline measurements were higher in early time points (0-10 minutes after injection) than the diet groups (**D**).

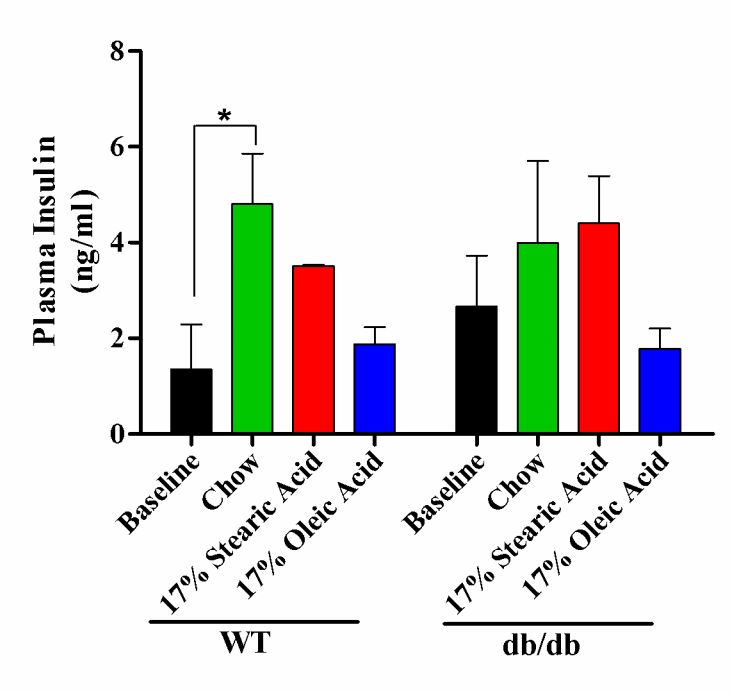


Figure 3.3.6: Plasma insulin content did not change with a moderate fat diet.

Insulin content was measured by ELISA and no significant difference was found between diet groups or genotypes. * $p<0.05$.

Chapter 4: A high fat diet enriched in stearic acid partially reversed hyperglycemia in db/db mice with prolonged hyperglycemia.

4.1 Introduction

In humans, Type 2 Diabetes Mellitus is more likely to be caused by obesity and lifestyle choices than genetic mutation such as the case in db/db mice. In humans, mutations in either the leptin protein (ob/ob) or the leptin receptor (db/db) are rare and are not sufficiently frequent to explain the increased rate of type 2 diabetes incidence in the last 50 years. Dietary influences and obesity are the largest risk factors in the development of type 2 diabetes. Most cases of type 2 diabetes can be directly linked to obesity and evaluating the amount and types of fat in the diet can offer an early treatment for both obesity and type 2 diabetes.

Clinically, no treatment of diabetes begins until clinical manifestations of the disease are present, well after hyperglycemia is established. In many cases, uncontrolled hyperglycemia may be present for months to years before diagnosis and treatment begin. Therefore, I examined if our experimental diets would be beneficial in slowing and reversing the symptoms of type 2 diabetes if started after diabetic symptoms were well pronounced.

I already observed the slowed progression of diabetic symptoms by the 40% kcal high fat stearic acid enriched diet in db/db mice. Thus, I tested if dietary stearic acid could rescue mice with established severe hyperglycemia. Since I already determined that db/db mice fed chow diet for ten weeks have a two fold increase in blood glucose levels from baseline, I maintained mice on chow diet before switching them to one of our experimental diets. In this study, I tested all four of our experimental diets in order to

determine: 1) if stearic acid affects blood glucose levels of mice with established hyperglycemia; 2) if there is a dose effect in the administration of stearic acid. Wild-type and db/db mice were fed the chow diet for six, eight, or ten weeks to establish hyperglycemia. The mice were then switched to 40% stearic acid, 17% stearic acid, 40% oleic acid, or 17% oleic acid diet for an additional six weeks of feeding. At the initiation of the diet and at the conclusion of the study, I measured fasting blood glucose, weight, and plasma insulin levels.

4.2 Materials and Methods

4.2.1 Animals

Wild-type, age-matched, male C57BLKS/J (WT, n=72) and BKS.Cg-*Dock7*^{m} +/+ *Lepr*^{db}/J (db/db, n=72) were purchased from The Jackson Laboratory (Bar Harbor, Maine) at four weeks of age. Upon receipt they were acclimated for one week to baseline conditions of a 12-hr light/dark cycle at 25°C on an *ad libitum* diet of commercially available standard rodent chow diet (2018 Teklad Global 18% Protein Rodent Diet; Harlan Laboratories, Madison, Wisconsin). At five weeks of age, I weighed the mice, gave each an ear tag, drew blood for a baseline blood glucose measurement, and randomly assigned each mouse to a diet group (n=8 mice per group per genotype). The diets were the standard chow diet and four experimental diets (40% kcal stearic acid diet Harlan Teklad TD.04096, 40% kcal oleic acid diet TD.09055, 17% kcal stearic acid diet, Harlan Teklad TD.03459, 17% kcal oleic acid diet TD.09315). Mice were fed *ad libitum*. All mice were fed standard chow diet until 6, 8, and 10 weeks of age to establish hyperglycemia. The mice were then switched to one of the four experimental diets for six additional weeks. **Figure 4.2.1** outlines the experimental design for this study. Animal

care and housing were conducted according to the NIH *Guide for the Care and Use of Laboratory Animals* and all experiments were approved by the Institutional Animal Care and Use Committee at the University of Kentucky.

4.2.2 Diets

Table 4.2.1, 4.2.2, and **4.2.3** list the nutritional value and composition of each diet used in this study. The stearic and oleic acid diets used in this study contained similar percentages of protein and carbohydrate whereas chow diet contained slightly more of total kcal from protein and 60% total kcal from carbohydrates. The fat content of each diet was unique. The experimental diets were custom made by Harlan Teklad using a modified TD.03459 diet. A Harlan Teklad nutritionist calculated all dietary nutritional values.

4.2.3 Glucose Measurements

Blood glucose was measured at the initiation of the diet (Baseline) and again at the end of the diet study. Mice were weighed and then fasted for four hours in a clean cage prior to testing. Fasting blood glucose was measured by tail prick with a commercially available glucometer and test strips (One Touch Ultra Glucose Monitoring Kit, Lifescan, Milpitas, California).

4.2.4 Body Weight

Weight was measured in conscious mice at the initiation of diet and at the completion of the diet study (6, 8, 10week groups) using a standard portable laboratory scale (Ohas, Model HH320, Fisher Scientific, Pittsburgh, Pennsylvania).

4.2.5 Insulin ELISA

Collected blood from fasted animals (4 hour fast) was allowed to clot for 20 minutes in a vacutainer and centrifuged at 1500 X g for 10 minutes to isolate serum. Serum was then snap-frozen and stored at -80^{0}C until analyzed. Plasma insulin levels were measured using a commercially available mouse/rat insulin ELISA (Millipore, Billerica, Massachusetts) and reported in ng/mL.

4.2.6 Data Analysis

Values are depicted as mean $\pm$ standard error and considered significant if $p < 0.05$. Data were statistically analyzed using two-way ANOVA with Bonferroni correction or one-way ANOVA with Dunnett's post hoc test when appropriate using GraphPad Prism 5.01 for Windows (GraphPad Software, San Diego, California). NIS-Elements 3.0 (Nikon Instruments, Elgin, Illinois) was used for islet area and staining analysis.

4.3 Results

4.3.1. Blood glucose levels in diabetic mice switched to a high fat diet enriched in stearic acid are lower than before the mice started the experimental diet.

Since I have already determined that db/db mice fed chow diet for ten weeks have a two fold increase in blood glucose levels from baseline, I designed a rescue study to examine the effects of stearic acid and oleic acid on blood glucose levels after hyperglycemia is established, similar to the onset of treatment in human type 2 diabetes. WT and db/db mice were fed the chow diet for 6, 8, or 10 weeks to establish hyperglycemia. The mice were then switched to 40% stearic acid, 17% stearic acid, 40% oleic acid, or 17% oleic acid diet for an additional six weeks. Blood glucose levels were tested at the initiation of the diet (Chow) and the cessation of the diet study. Wild-type

mice switched from the chow diet to any of the experimental diets maintained normal blood glucose levels regardless of how long they were fed chow diet (**Figure 4.3.1A, B, C**).

Db/db mice fed chow for 6 weeks and then switched to 17% oleic acid diet had decreased blood glucose levels compared with the baseline chow measurement (**Figure 4.3.1A**). Db/db mice switched to 17% stearic acid, 40% oleic acid, and 17% oleic acid diets continued to have elevated blood glucose levels after switching from chow following 8, and 10 weeks of feeding (**Figure 4.3.1A, B, C**). Db/db mice switched to 17% stearic acid diet after 6 weeks of chow feeding had decreased blood glucose compared to the chow baseline, 17% oleic acid, and 40% oleic acid fed mice. Additionally, the 40% stearic acid diet slowed and even reversed the progression of type 2 diabetes in diabetic mice with hyperglycemia regardless of time on chow.

4.3.2. Body weight was not changed by rescue diet intervention.

Wild-type mice fed chow diet for 6 weeks and then were switched to 40% stearic acid diet had significantly decreased body weight compared to all other diet groups and the baseline measurements (**Figure 4.3.2A**). After 8 weeks of chow feeding and switching to 40% stearic acid diet, wild-type mice had lower blood glucose than 17% stearic acid, 17% oleic acid, and 40% oleic acid fed mice but not lower than baseline glucose measurements (**Figure 4.3.2B**). No change in body weight was detected in mice that were fed chow diet for 10 weeks before being switched to 40% stearic acid diet (**Figure 4.3.2C**).

Diabetic mice fed chow diet for 6 weeks and then switched to 17% oleic acid and 40% oleic acid diets had increases in body weight as compared to baseline weight

measurement (**Figure 4.3.2A**). After 8 weeks of chow diet and then switching to 40% stearic acid diet, db/db mice had a significant decrease in body mass compared to 17% stearic acid, 17% oleic acid, and 40% oleic acid diets, but no different from baseline measurement (**Figure 4.3.2.B**). Importantly, db/db mice switched from chow diet to 40% stearic acid diet after 10 weeks of chow feeding had a large and significant decrease in blood glucose levels even though their body mass did not decrease significantly compared to mice fed chow diet (**Figure 4.3.2C**). Db/db mice fed 17% stearic acid, 17% oleic acid, and 40% stearic acid diets had no significant changes in body weight (**Figure 4.3.2C**).

4.3.3. Plasma insulin was not changed by rescue diet.

Plasma insulin content was measured only in the 10 week study. There were no significant differences in plasma insulin content in any of the diet groups or genotypes.

4.4 Discussion

Dietary interventions for treating hyperglycemia have been relatively ineffective at maintaining lowered blood glucose for a significant duration of time. Most of these dietary interventions focus on weight loss as a means to also decrease blood glucose. In most clinical manifestations of type 2 diabetes, patients are often hyperglycemic for long periods of time (months to years) before seeking treatment. Diet induced obesity is linked to high intake of dietary fat. Combining a high fat diet with hyperglycemia may exacerbate hyperglycemic conditions. In this study, I examined whether a high fat dietary intervention in which I modified the type of fat consumed could lower blood glucose levels in mice with established and severe hyperglycemia. I found that a high fat diet (40% kcal from fat) enriched in stearic acid reduced blood glucose levels without a

change in weight. However, a low fat diet (17% kcal from fat) enriched in stearic acid had no effect on blood glucose levels in db/db mice with established hyperglycemia. Similarly, mice fed a low fat diet with mixed fats (normal chow diet) and mice fed a high fat diet enriched in oleic acid, a mono-unsaturated fat, did not reverse or slow the progression of hyperglycemia. The mice fed these diets continued to have high blood glucose levels. These data indicate that a high-fat diet enriched in stearic acid may be a suitable dietary intervention for treating hyperglycemia, especially in patients who have trouble adhering to a low fat diet.

Table 4.2.1: Dietary Components

	Catalog Number	Protein	Carbohydrate	Fat
Chow	TD. 2918	18.80	53.80	17.0
40% kcal Stearic Acid	TD. 04096	17.80	42.20	40.0
40% kcal Oleic Acid	TD. 09088	17.50	41.50	41.0
17% kcal Stearic Acid	TD. 03459	19.00	64.40	16.60
17% kcal Oleic Acid	TD. 09315	18.80	63.90	17.20

Table 4.2.2: 40% diet composition

Diet	Chow	Stearic Acid	Oleic Acid
% kcal from Fat	17.00	40.00	41.00
% Stearic acid in fat	2.60	86.00	2.00
% Oleic acid in fat	22.50	<1.00	64.00
% Other Essential Fatty Acids	74.90	13.00	34.00

Table 4.2.3: 17% diet composition

Diet	Chow	Stearic Acid	Oleic Acid
% kcal from Fat	17.00	16.60	17.20
% Stearic acid in fat	2.60	85.00	2.00
% Oleic acid in fat	22.50	1.00	65.00
% Other Essential Fatty Acids	74.90	14.00	34.00

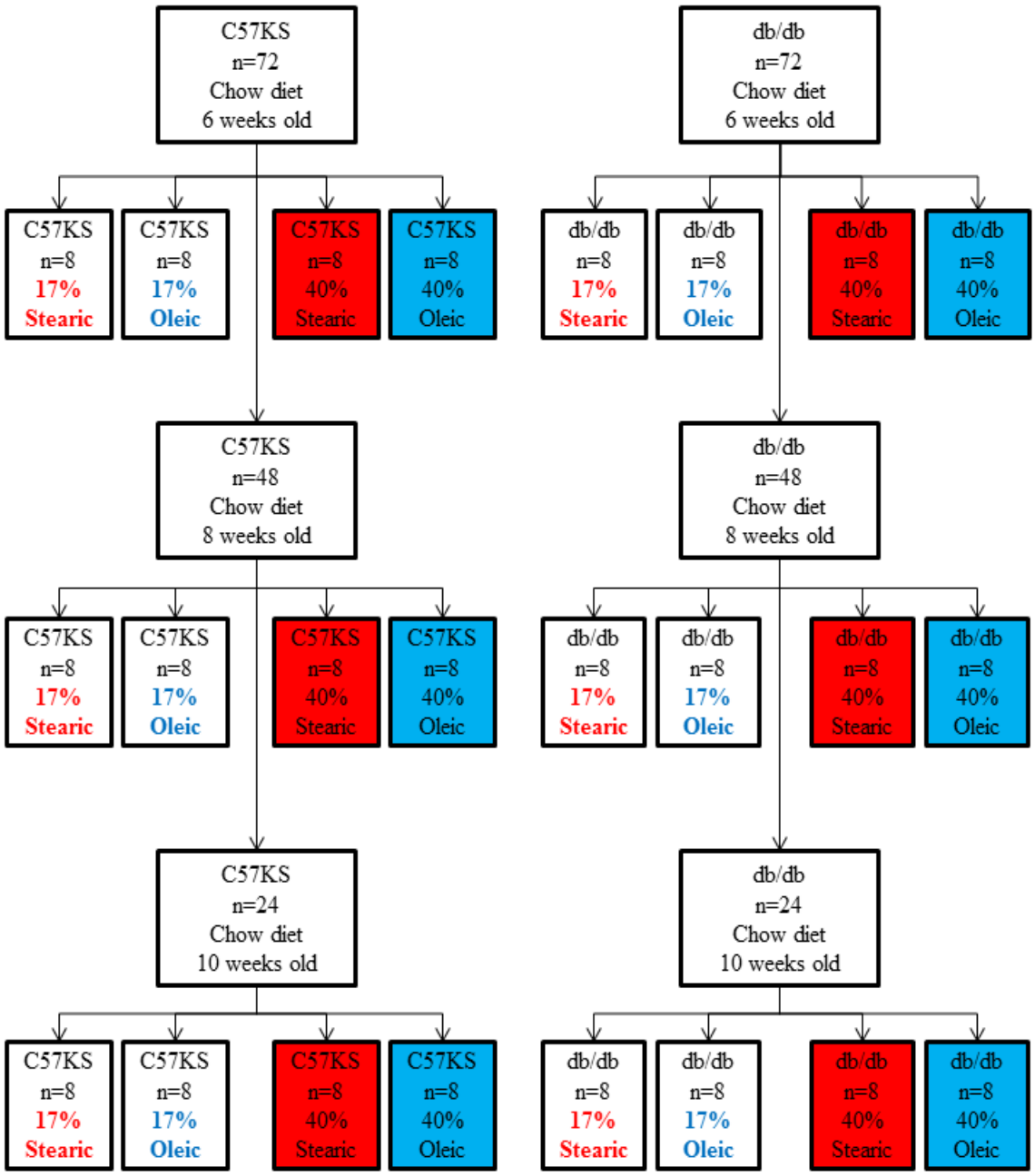

C57KS
n=72
Chow diet
6 weeks old
db/db
n=72
Chow diet
6 weeks old
C57KS
n=8
17%
Stearic
C57KS
n=8
17%
Oleic
C57KS
n=8
40%
Stearic
C57KS
n=8
40%
Oleic
db/db
n=8
17%
Stearic
db/db
n=8
17%
Oleic
db/db
n=8
40%
Stearic
db/db
n=8
40%
Oleic
C57KS
n=48
Chow diet
8 weeks old
db/db
n=48
Chow diet
8 weeks old
C57KS
n=24
Chow diet
10 weeks old
db/db
n=24
Chow diet
10 weeks old

Figure 4.2.1: Experimental design for rescue diet study. In a separate but parallel study to examine the effects of dietary stearic acid on adult mice with severe and prolonged hyperglycemia, db/db and WT mice were aged to 6 weeks, 8 weeks, and 10 weeks of age on standard chow diet. These mice were then switched to one of the four experimental diets: 17% kcal stearic acid, 17% kcal oleic acid, 40% kcal diet stearic acid, or 40% kcal oleic acid and maintained on these experimental diets for 6 weeks. Similar parameters from the previous studies were measured: blood glucose, weight, and body composition.

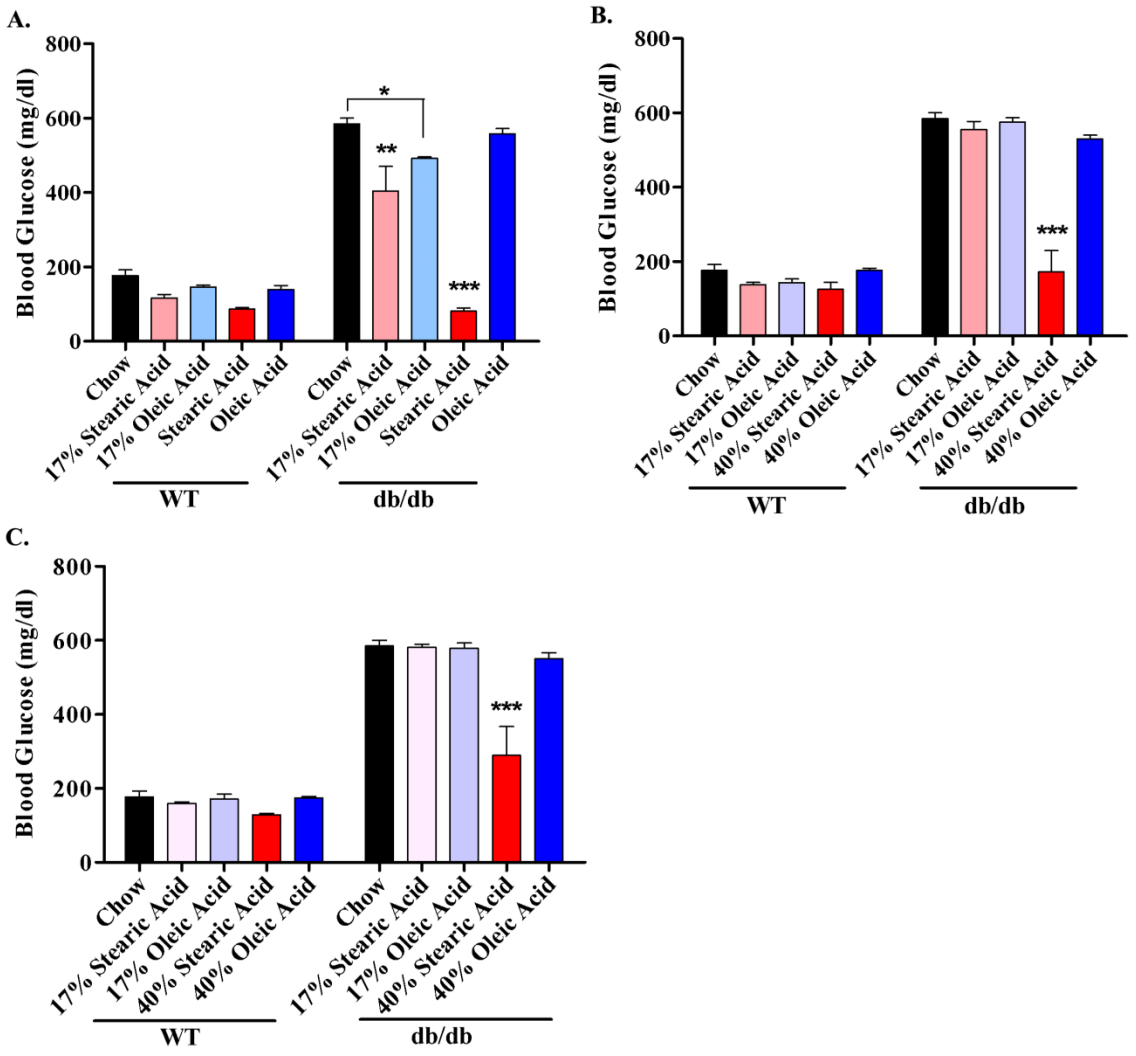


Figure 4.3.1: A high fat diet enriched in stearic acid decreases blood glucose levels in db/db mice with pre-existing hyperglycemia. A. 6 weeks chow → 6weeks experimental diet. **B.** 8 weeks chow → 6 weeks experimental diet. **C.** 10 weeks chow → 6 weeks experimental diet. * $p<0.05$, * $p<0.01$, *** $p<0.001$, n=8 per group.

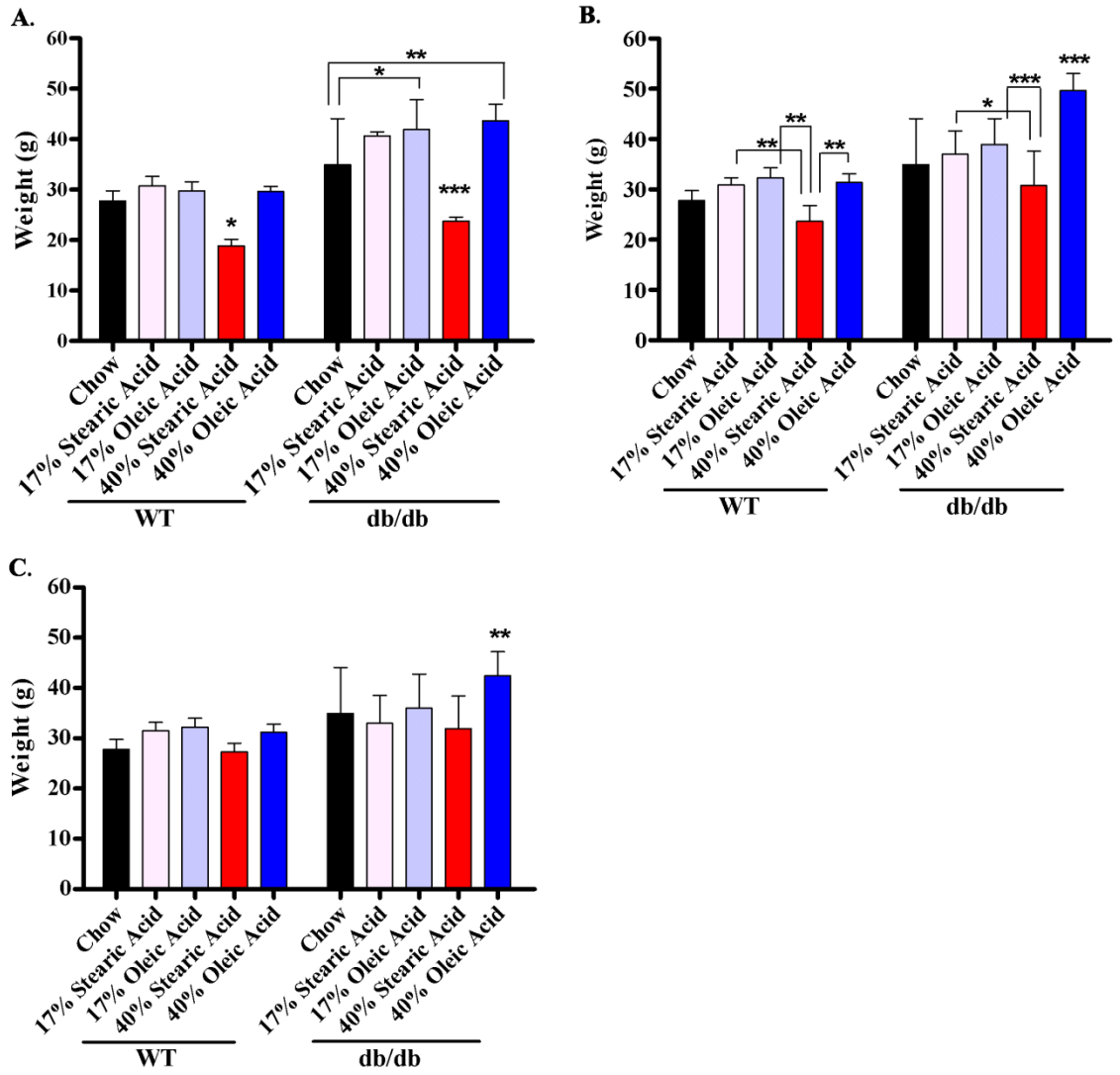


Figure: 4.3.2: Body mass during rescue diet study. A. 6 weeks chow→6 weeks experimental diets. db/db mice switched from a chow diet to a 17% and 40% fat diet enriched in oleic acid have increased weight compared to baseline (chow). Both WT and db/db mice fed 40% stearic acid diet had decreased body weight after **B.** 8 weeks chow→6 weeks experimental diets. WT and db/db mice 40% stearic acid diet had lower body mass than other diet groups but not lower than baseline weight. **C.** 10 weeks chow→6 weeks experimental diets. Db/db mice fed 40% oleic acid diet had significantly

higher body mass than db/db mice fed any other diet and higher than baseline. *$p<0.05$, **$p<0.01$, ***$p<0.001$ and n=4-8 per diet group per genotype.

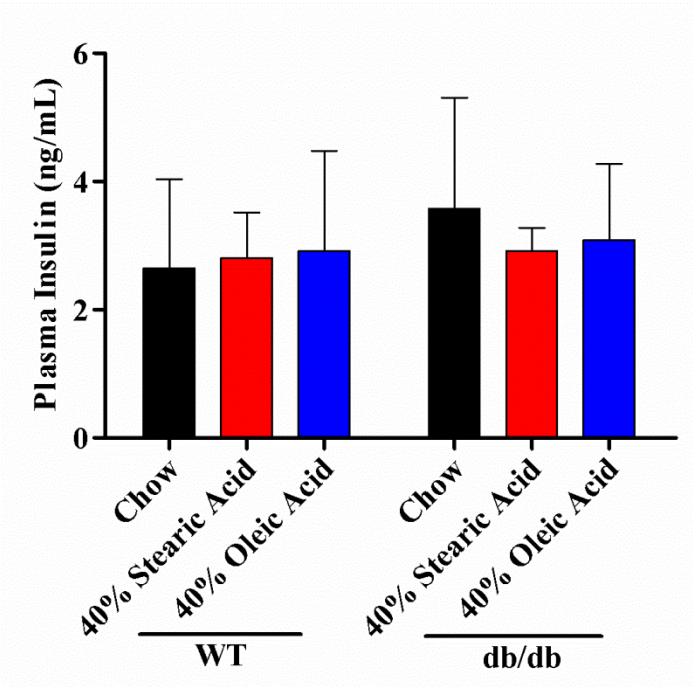


Figure 4.3.3: 40% high fat diet enriched in stearic acid or oleic acid has no effect on plasma insulin levels. Plasma insulin levels of mice placed on 40% high fat diets at ten weeks of age were measured after 6 weeks on diet. There were no differences in plasma insulin levels in any group of mice.

Chapter 5: Discussion

The purpose of this research was to test the hypothesis that a high fat diet enriched in stearic acid slows the progression of type 2 diabetes in a db/db mouse model. The work presented in this dissertation supports my global hypothesis that stearic acid has protective effects in slowing the progression of type 2 diabetes. These findings are novel in the field of type 2 diabetes dietary fatty acid research and may improve upon current dietary recommendations for diabetes patients.

Chapter 2 examined the effects of a high fat diet enriched in stearic acid on the progression of type 2 diabetes in a diabetic mouse model. Using this murine model and experimental diet, evidence was provided that the progression of type 2 diabetes was slowed when mice were fed a high fat diet enriched in stearic acid during the diet study. Additionally, the db/db mice were protected from severely elevated blood glucose levels, a major factor in pancreatic beta cell failure; this protection was unrelated to weight loss (100). This supports the hypothesis that the effects of stearic acid are due to alterations in fatty acid absorption in the gut. The mechanism by which stearic acid has decreased absorption is unknown. However, the decreased absorption does support the idea that stearic acid is beneficial in slowing the progression of type 2 diabetes. In this study, I found that a high-fat diet enriched in stearic acid, but not a high-fat diet enriched in oleic acid, a monounsaturated fat, was able to lower blood glucose levels in db/db mice without a concomitant change in body weight, a novel finding in diabetic diet studies.

In Chapter 3, I asked whether stearic acid has beneficial effects in a low fat diet, a regimen more closely aligning with current dietary recommendations (~20% kcal from fat). Lowering the fat content in the experimental diets from 40% kcal from fat to 17%

kcal from fat significantly impaired the effectiveness of the diet as compared to the 40% kcal experiments. Blood glucose levels in wild-type mice did not differ from those of db/db mice on the moderate fat diets. Db/db mice fed chow, stearic acid and oleic acid enriched diets all and increased blood glucose levels. This result indicates that stearic acid has a dose-dependent effect on blood glucose levels and additional studies are warranted to investigate the amount of fat absorbed in this moderate fat diet. Since stearic acid has no detrimental effect on cholesterol levels, it may be incorporated into the diet as a treatment for lowering blood glucose levels without concern for cardiovascular complications. I found that only a high-fat diet enriched in stearic acid was able to control hyperglycemia in db/db mice. Mice fed moderate-fat diets (17% kcal from fat) enriched in stearic or oleic acid were not as fat as the high-fat fed mice, but had blood glucose measurements that were higher than their baseline measurements.

Chapter 4 extended the hypothesis that stearic acid has beneficial blood glucose lowering effects and focused on treating mice that had established severe hyperglycemia. I designed a 'rescue' diet study in which wild-type and db/db mice were fed chow diet for 6, 8, or 10 weeks before being switched to one of the experimental diets. I confirmed the results from the first and second experiments: a high-fat diet (40%kcal from fat) lowered blood glucose levels without a decrease in weight; a moderate fat diet (20%kcal fat) did not lower blood glucose levels in db/db mice. Further I found that only a diet enriched in stearic acid, not the unsaturated fat oleic acid, was able to lower blood glucose levels. The ability of a stearic acid enriched high fat diet to slow the progression of diabetes and reverse hyperglycemia in db/db mice argues that risks and benefits of fats in the diet

depend on the species, rather than the chemical class, of fats ingested. The beneficial effect of stearic acid appears to be associated with a decreased absorption of dietary fat.

5.1 Future Directions

My first and second aims were designed to determine if stearic acid could lower blood glucose levels in diabetic mice and if there was a dose-dependent effect of stearic acid on blood glucose levels. I used direct dietary supplementation of stearic acid and evaluated the effects on the disease state to determine if a high-fat diet enriched in stearic acid, a saturated fat, slows the progression of type 2 diabetes in db/db mice. Diets enriched in saturated fats have been labeled as detrimental to overall health and allegedly linked to cardiovascular events. Previous studies have reported that dietary stearic acid has minimal effects on plasma cholesterol levels, unlike the saturated fat palmitic acid, another common dietary saturated fat, which increases LDL cholesterol. Stearic acid may be used as a dietary treatment option for type 2 diabetes without concern for detrimental cardiovascular effects.

In the work for the first and second aims, I found there was, in fact, a dose dependent effect of stearic acid. Db/db mice fed stearic acid had lower blood glucose levels when fed a high fat diet (40% kcal from fat) enriched in stearic acid but not when fed a 17% kcal moderate fat diet enriched in stearic acid. Stearic acid may have worked more efficiently at lowering blood glucose levels in the high fat diet than in the moderate fat diet because the amount of fat in the diet was able to overwhelm the fat transport system.

Stearic acid poses a unique problem for the fat emulsification, hydrolysis, and absorption pathways. Stearic acid, like many saturated fats is solid at room temperature

(10). Unlike saturated fats that have been tested in previous dietary studies, solid stearic acid is not emulsified or hydrolyzed efficiently by endogenous agents (109). To be absorbed from the diet, fats must be emulsified by bile salts made by the liver and stored in the gallbladder (143). The pancreas makes and secretes pancreatic lipase which enzymatically cleaves the fatty acids tails from the glycerol (143). Lipase, however, is water soluble and can only cleave fatty acids that are near the edge of the emulsion micelle (173). Although there is a large amount of pancreatic lipase in the small intestine ready to hydrolyze fats, it is also easy to visualize how a high-fat diet easily overwhelms the emulsification and hydrolytic processes. Ingesting large amounts of fat, especially saturated fat, requires large amounts of bile to complete emulsify the fats. A disruption in the fat and bile ratio leaves fat globules intact rather than emulsified in micelles (80, 178). The intact fat globules are not absorbed across the intestinal wall and instead move through the digestive tract for excretion. I have shown that stearic acid is not well absorbed and is excreted in the feces of the mice fed a diet enriched in stearic acid. Importantly, the mice fed a high-fat diet enriched in stearic acid excreted more fat in the feces than those mice fed a high-fat diet enriched in oleic acid, an unsaturated fat that is emulsified and hydrolyzed efficiently (77).

This demonstration of manipulation of the fat absorption pathway is not novel. The drug orlistat works by blocking pancreatic lipase thereby decreasing digestion and absorption of fat and increasing excretion (79). This mechanism of using a fat to block its own absorption and act in a manner similar to well defined pharmaceutical, orlistat, is novel and may have future promise in dietary treatments of type 2 diabetes instead of reliance on pharmaceuticals.

Similarly, stearic acid did not provide all the caloric value of the diet. 60% of the kilocalories in the diet came from protein and carbohydrate. The poor absorption of stearic acid from the gut eliminates the fat from providing a sufficient primary fuel source. Thus, the mice must utilize the remainder of the diet, the protein and carbohydrates, for fuel, effectively lowering the caloric content without restricting food consumption. The decrease in blood glucose levels did not occur until two weeks after the diet intervention was started indicates that the diet intervention had a compensatory time, during which the body, most notably the gut, was adjusting to the change in nutrient supply. This, together with the increase in fecal fat excretion and low liver accumulation of stearic acid, supports studies that have shown stearic acid may change the gut microflora (42, 158). The gut microflora, the resident bacteria of the gut, is also involved in metabolizing diet contents. The microflora constituents can change to adjust to the dietary demands placed upon it. Increasing the fat content will demand for more fat-metabolizing gut bacteria and less carbohydrate-metabolizing bacteria (32, 33). Examining the constituents of the gut microflora may indicate how mice were utilizing the diets administered to them.

Recall that carbohydrates are the preferred fuel source and can be easily metabolized; however, proteins, like fat, are not as efficiently metabolized. To test if the mice were utilizing the other diet components in place of the fat, one may measure the waste product from the metabolism of protein: urea (97). Elevated levels of urea in the liver and urine of db/db mice on stearic acid diet compared to WT and db/db mice on non-stearic acid diets would indicate that the stearic acid fed mice were using proteins as a primary fuel source and were able to maintain their body weight by using the

components in the diet more efficiently than the db/db mice fed oleic acid and chow diets that contained fat that was easily absorbed.

Though I found that stearic acid was not well absorbed by the mice, there is little doubt that the mice are absorbing some of the stearic acid from the diet. Stearic acid may have effects on the insulin release signaling cascade. Stearic acid, similar to other long-chain fatty acids, acts as a cellular signaling molecule in the insulin release pathway (69, 70). The G-protein coupled receptor, GPR40, which is expressed in the pancreatic beta cells, plays a role in potentiating the insulin release pathway and has a fatty acid binding domain (26, 95, 167). Stearic acid has the potential to bind to the fatty acid binding domain of GPR40 thereby activating the receptor and downstream signaling cascade culminating in insulin granule fusion and insulin release from the pancreatic beta cells.

Given the mechanism outlined above, the input of stearic acid on the GPR40 pathway should be evaluated. Steneberg et al. (164) previously showed that GPR40 knockout mice were resistant to fatty-acid modulation of insulin secretion and normoinsulinemic. Additionally, GPR40 overexpressing transgenic mice were hypoinsulinemic and diabetic (56). My data point toward fatty-acid potentiation of insulin secretion and intense contribution of fatty-acid to insulin resistance though the GPR40 pathway.

Steneberg et al. did not control the specific fats in the diet; mice were fed either a high-fat, mixed fat diet or a control diet (164). To evaluate the effects of stearic acid on GPR40 activation, the Steneberg study could be replicated to include the stearic acid presented in this work. Measurements of plasma insulin, glucose tolerance and insulin tolerance would evaluate the effect of stearic acid on the GPR40 pathway. If stearic acid

influenced the GPR40 pathway, one would observe an increase in plasma insulin levels, no glucose or insulin intolerance. If stearic acid did not contribute to the GPR40 pathway, insulin levels would increase, and both glucose and insulin tolerance would decrease. Additionally, GPR40 content in the pancreas should also be examined. If there were no change in the amount of GPR40 present in the islets from WT mice, overactivation of GPR40 would not be likely.

Similarly, caveolin-1, a membrane bound protein found abundantly in the pancreatic beta cells that has a role in insulin secretion, acts as a gate in the insulin granule fusion and release pathway (138). Insulin granule fusion with the plasma membrane is controlled by the small Rho family GTPase, cdc42, in complex with VAMP2, and by caveolin-1 (138). When associated with caveolin-1, the granules do not fuse with the membrane (138). However, upon glucose stimulation, caveolin-1 releases the inhibition on the cdc42-VAMP2, and the insulin granules fuse with the membrane to release insulin (138). Moreover, a fatty acid, like stearic acid, could bind to caveolin-1, and, via the cdc42 pathway, increase insulin secretion by releasing the inhibition of caveolin-1 on the insulin granules (169).

In the pancreatic islets, fatty acids, such as stearic acid, may bind to caveolin-1 and move the fatty acid into the cell while simultaneously releasing the inhibition from insulin granules. This would increase pancreatic islet fatty acid content and increase insulin exocytosis. To test modulation of the insulin granule pathway by stearic acid, a few experiments would be effective. Isolated pancreatic islets from diet treated WT and db/db mice would be measured for fatty acid content by GC/MS. Next, another set of islets would be incubated with stearic acid in the culture media. The culture media would

be collected and analyzed for secreted insulin content and fatty acid by ELISA and GC/MS, respectively. Additionally, the islets would need to be homogenized and analyzed for stearic acid content. If insulin content of the culture media in the islet from the diabetic animals increases and stearic acid content decreases, stearic acid may positively modulate the insulin secretion pathway by removing the inhibition of caveolin-1 on the insulin granules. Simultaneously, stearic acid may bind to caveolin-1 for transport into the islets resulting in a higher stearic acid concentration inside the pancreatic islets. Removal of fatty acids from the culture media would ameliorate this effect.

In only one cohort I observed a decrease in weight in the db/db mice fed stearic acid. The maintenance of weight coupled with lower blood glucose is contradictory to most diabetic dietary intervention studies (45, 104). The experimental diets, both stearic acid and oleic acid diets, had similar compositions of protein and carbohydrate components and only differed in the type of fat, not the amount of fat, that was added. The maintenance of weight in the stearic acid diet group may indicate better glucose uptake by the skeletal muscle.

Another mechanism by which stearic acid may have lowered blood glucose levels in the diabetic mice is through improvements in skeletal muscle uptake of glucose. Increasing glucose uptake into the skeletal muscle would decrease blood glucose levels (11, 52, 128). Glucose uptake into the skeletal muscle is driven by the activation of the insulin receptor. Insulin binding its receptor activates a signaling cascade that moves more GLUT-4 glucose transporters onto the cell membrane (44). Glucose moves through the glucose transporter and into the muscle cell (106). Caveolin-1 also plays a role in

insulin receptor activation. Previous studies have reported that insulin can activate caveolin-1 by phosphorylation at tyrosine 14 (157). Activation of caveolin-1 has been reported in increase GLUT-4 translocation (36, 120). My results indicated that db/db mice fed a high fat diet enriched in stearic acid did not have decreased plasma insulin levels; therefore, the circulating insulin could bind to its receptor activating caveolin-1 to promote enhanced GLUT-4 translocation. Additionally, the increased level of circulating fatty acids may promote additional caveolin-1 to the plasma membrane. Additional caveolin-1 on the membrane may have the potential to further increase the translocation of GLUT-4 to the skeletal muscle membrane. An increased amount of caveolin-1 would also result in an increase in lipid deposit in the muscle cell. Immunohistological staining for GLUT-4 on the muscle cell surface as well as skeletal muscle lipid content identified by Oil Red O staining would indicate if stearic acid is promoting lower blood glucose levels by increasing glucose transport into the skeletal muscles.

The work presented in this dissertation supports my global hypothesis that stearic acid has protective effects in slowing the progression of type 2 diabetes. These findings are novel in the field of type 2 diabetes dietary fatty acid research and may improve upon current dietary recommendations for diabetes patients.

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Vita

Valerie Lynn Reeves
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Education

Aug 2003- May 2007	Austin Peay State University Bachelors of Science in Biology
Aug 2007-April 2012	University of Kentucky Doctoral Candidate in the Department of Physiology. First year in the Integrated Biomedical Science program (IBS)

Research Experience

Aug 2005- Apr 2006	Independent Study Research in the lab of Dr. Sergei A. Markov (Department of Biology, Austin Peay State University) Investigated biological hydrogen production by E. coli.
Aug 2007- Oct 2007	IBS Research Rotation in the laboratory of Dr. Susan Smyth (Department of Physiology, University of Kentucky) Examined the role of signaling targets in platelet adhesion.
Oct 2007- Dec 2007	IBS Research Rotation in the laboratory of Dr. Craig Miller (Department of Microbiology, Immunology and Molecular Genetics, University of Kentucky) Studied the role of TNFβ in herpes simplex I virus.
Jan 2008- May 2008	IBS Research Rotation in the laboratory of Dr. Eric Smart (Department of Physiology, University of Kentucky) Investigated caveolin-1 localization and extraction in various murine tissues.
May 2008- Aug 2010	Graduate Student in the laboratory of Dr. Eric Smart (Department of Physiology, University of Kentucky) Investigated the role of dietary stearic acid in alleviating hyperglycemia associated with type 2 diabetes.
Aug 2010- Apr 2012	Doctoral Candidate under the direction of Dr. David C. Randall and Dr. Timothy S. McClintock (Department of Physiology, University of Kentucky) The role of dietary stearic acid in alleviating hyperglycemia associated with type 2 diabetes in a diabetic mouse model

Coursework

- Human Physiology
- Principles of Systems, Cellular, and Molecular Physiology
- Biomolecules and Metabolism
- Cell Biology
- Experimental Genetics
- Biomolecules II
- Cell Signaling
- Integrated Biomed Systems
- Seminar Tutorial in Physiology
- Graduate Seminar in Physiology
- Ethics in Scientific Research
- Grant-writing Workshop

Publications

Reeves VL, Thomas CM, Smart EJ. 2010. Lipid Rafts, Caveolae and GPI-Linked Proteins. *Caveolins and Caveolae: Roles in Signaling and Disease Mechanisms.* Editors: Jasmin, JF and Lisanti, ML.

Reeves VL, Thomas CM, McClintock, TS, Randall DC. 2012. Dietary fat absorption and the effects on blood glucose in diabetic (db/db) mice. American Journal of Physiology - Metabolism (in preparation)

Thomas, C.M., **Reeves, V.L.**, Bhatnagar, S., Goulding, D.S., Sudduth, T.S., Randall, D.C. 2012. A diet enriched in stearic acid inhibits the development of diabetic cardiomyopathy in db/db mice. (in preparation)

Presentations

2008 – 2009 Physiology Guttman Seminar Series
Fall 2009 Physiology Departmental Seminar
Spring 2010 Physiology Departmental Seminar
April 2012 Physiology Departmental Defense Seminar

Abstracts and Poster Presentations

Reeves, VL, Smart EJ. 2009. The effects of fatty acids on insulin secretion. *FASEB J.* April 2009; 23:990.34. Experimental Biology 2009.

Reeves, VL, Smart EJ, McClintock TS, Randall DC. 2010. A link between lower blood glucose and dietary stearic acid intake in diabetic mice. Published online December 2010. Keystone Symposium, Keystone, CO. January 2011.

Reeves, VL, Thomas, CM, McClintock, TS, Randall, DC. 2011. A link between lower blood glucose and dietary stearic acid intake in diabetic mice. University of Kentucky Endowed Professor Program Day, Lexington, KY. March 2011

Reeves, VL, Randall, DC. 2011. Dietary Fat and Type 2 Diabetes: The Good, the Bad and the Ugly. Barnstable-Brown Diabetes and Obesity Research Day, Lexington, KY. May 2011

Reeves, VL, Randall, DC. 2011. A Diet Enriched in Stearic Acid Slows the Progression of Type 2 Diabetes in Mice. Gill Heart Research Day, Lexington, KY. October 2011

Awards and Honors

2007-2012	Graduate Research Assistantship, Integrated Biomedical Sciences Program, University of Kentucky
2009	University of Kentucky Graduate School Travel Award
2011	University of Kentucky Cardiovascular Training Grant T-32 (awarded but declined)

Positions

2010- Present	Adjunct Faculty Bluegrass Community and Technical College, Lexington, KY BIO 130: Aspects of Human Biology lecture instructor, BIO 137: Anatomy and Physiology I lecture and laboratory instructor, BIO 139: Anatomy and Physiology II, laboratory instructor
2009-2010	Student Leader University of Kentucky, Department of Physiology Teaching, Education and Mentoring (TEaM). Student run organization that focuses on providing an environment to explore career options by inviting speakers in all science-based fields, hosting workshops to complete teaching portfolios and curriculum vitas, and host discussions about mentoring concerns.

Society Memberships

2007 - Present	American Association for the Advancement of Science (AAAS)
2010 - Present	American Diabetes Association
2012 – Present	American Heart Association
2011 – Present	American Physiological Society
2011 – Present	Graduate Women in Science, Bluegrass Chapter (Beta Chi)