# Do Dietary Fats Contribute to Fatty Liver: Impact of Linoleic Acid Rich Oil on Liver Fat and Fatty Acid Composition in Diet-Induced Obese Mice

Thesis

Presented in Partial Fulfillment of the Requirements for the Degree Master of Science in the Graduate School of The Ohio State University

By

Connor Mahler

Graduate Program in Human Nutrition

The Ohio State University

2021

Thesis Committee

Dr. Martha Belury - Advisor

Dr. Amanda Bird

Dr. Joshua Bomser

# Abstract

Non-alcoholic fatty liver disease (NAFLD) is a common occurrence in people with insulin resistance and NAFLD increases the risk of developing liver disease. Although the pathogenesis of NAFLD is not well understood, recent evidence suggests that mitochondria dysfunction is critical in its development. A dominant species of cardiolipin in the inner mitochondria membrane is tetra-linoleoyl-cardiolipin ($LA_4CL$), a phospholipid with molecules of linoleic acid (LA). Reduced levels of $LA_4CL$ are linked with mitochondrial dysfunction. The hepatic phospholipid fatty acid profiles may reflect both the disease of NAFLD as well as dietary fat composition.

The overall hypothesis of my thesis is: consumption of a linoleic acid (LA) rich oil will alleviate progression of fatty liver in mice by increased incorporation of LA into hepatic phospholipids. To test this hypothesis, we fed mice two different high fat diets, one containing a LA rich safflower oil and the other diet containing lard. To determine any changes in hepatic fat accumulation we measured triglyceride (TG), and total lipid content. Phospholipid fatty acid composition was assessed as well to determine any changes in phospholipid fatty acids.

C57BL6/J male mice (N=24) were assigned to a high fat diet of 24% wt fat containing either LA-rich safflower oil or lard which is rich in saturated fat, for 130 days. Body composition, blood glucose and insulin sensitivity measurements were assessed.

Solid phase extraction (SPE) and gas chromatography were used to analyze the fatty acid composition of the hepatic phospholipids; HPLC/MS in collaboration with Dr. Genevieve Sparagna (University of Colorado) was used to determine cardiolipin speciation.

An insulin tolerance test revealed that blood glucose remained lower at 90 min and 120 min after the insulin injection in the SO diet compared to the LD diet. The SO diet had increased the percent of $LA_4CL$ compared to the LD diet of total cardiolipin species. No differences in total lipids or TG in the liver between diets. Of the phospholipid fractions extracted, the fatty acid compositions reflected major species of fatty acids found in their respective diets in the phospholipid fractions reported. This study indicates that dietary fat altered fatty acid compositions of phospholipids, and consumption of a high fat LA-rich safflower oil does not alleviate fatty liver accumulation in mice fed a high fat diet.

# Acknowledgments

I would like thank Dr. Belury, for her mentorship, advice, and support throughout my graduate years here at Ohio State. I am grateful for being able to work with her, including the opportunity to help lead a pilot study.

I would like to thank Rachel Cole, for all help and immense support with data organization and collecting data for this project. Thank you for working with me and teaching me lab skills that I can bring into the next steps in my career. Thank you for inspiring me to become a better researcher, and scientist.

I would like to thank Deena Snoke and Austin Angelotti for all their support and help throughout my project and being a consistent support system for all my many questions during my project's duration. Thank you both for inspiring me to become a better researcher and scientist.

Thank you to both past and present Belury Lab students that I have gotten to know – Evelyn, Aimee, Ariel. While we have met remotely this year it is still nice to visit and catch up with everyone during lab meeting.

I want to thank all the graduate students and OSUN professors both past and present, as it has been a wonderful opportunity to work and get to know many talented and diverse graduate students and professors here at Ohio State.

I want to thank all my friends, family members for their support during my graduate career.

I would like to thank my committee, Dr. Belury, Dr. Bird and Dr. Bomser for their time, and help.

# Vita

2013..... Lima Central Catholic

2017..... B.S. Nutrition Science, The Ohio State University

2017 to 2019 ..... Nutritional Aide, Wexner Medical Center, The Ohio State University

2019 to present ..... Graduate Teaching Associate, Department of Human Nutrition, The Ohio State University

Fields of Study

Major Field: Human Nutrition

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# List of Abbreviations

| | |
|---|---|
| NAFLD | Non-alcoholic Fatty Liver Disease |
| LA | Linoleic Acid |
| L4CL | Tetra-linoleoyl Cardiolipin |
| MetS | Metabolic Syndrome |
| NASH | Non-alcoholic steatohepatitis |
| AA | Arachidonic Acid |
| TAG | Triglycerides |
| DAG | Diglycerides |
| BMI | Body Mass Index |
| T2DM | Type Two Diabetes |
| TNF-α | Tumor Necrosis Factor Alpha |
| MCP-1 | Monocyte Chemoattractant Protein-1 |
| VAT | Visceral Adipose Tissue |
| SAT | Subcutaneous Adipose Tissue |
| IL-6 | Interlekin-6 |
| IRS-1 | Insulin Receptor Substrate 1 |
| PPAR-α | Peroxisome Proliferator-activated Receptor Alpha |
| T2DM | Type 2 Diabetes |
| PGC-1α | Peroxisome Proliferator-Activated Receptor-Gamma Coactivator |
| NRF-2 | Nuclear Respiratory Factor-2 |
| TFAM | Transcription Factor A, Mitochondrial |
| PA | Palmitic Acid |
| SA | Stearic Acid |
| PUFAS | Polyunsaturated Fats |
| PPARβ/δ | Peroxisome Proliferator-activated Receptor Beta/Delta |
| PPARγ | Peroxisome Proliferator-activated Receptor Gamma |

| | |
|---|---|
| MUFAs | Monounsaturated Fatty Acids |
| HbA1c | Hemoglobin A1c |
| TAZ | Taffazin |
| IMM | Inner Mitochondrial Membrane |
| PA | Phosphatic Acid |
| CDP-DAG | CDP Diacylglycerol |
| CDS | CDP-DAG Synthase |
| PG | Phosphatidylglycerol |
| CLS | Cardiolipin Synthase |
| MLCL | Monolysocardiolipin |
| MLCLAT-1 | MLCL Acyltransferase |
| ALCAT-1 | Lysocardiolipin Acyltransferase |
| PC | Phosphatidylcholine |
| PE | Phosphatidylethanolamine |
| PI | Phosphatidylinositol |
| PS | Phosphatidylserine |
| CK | Choline Kinase |
| CCT | Phosphocholine Cytidylyltransferase |
| CPT | Choline Phosphotransferase |
| EK | Ethanolamine Kinase |
| ECT | Phophoethanolamine Cytidylyltransferase |
| EPT | CDP-ethanolamine phosphotransferase |
| PIS | Phosphatidylinositol Synthase |
| MAM | Mitochondrial Associated Membrane |
| DHA | Docosahexaenoic Acid |
| OA | Oleic Acid |

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# Chapter 1. Introduction

Non-alcoholic Fatty Liver Disease (NAFLD) is defined as the accumulation of fat in the liver in patients that do not consume alcohol (1). Currently estimates are that NAFLD affects 30% of the US population and many of these individuals exhibit characteristics of Metabolic Syndrome (MetS) (2). NAFLD encompasses a wide spectrum of conditions from simple fat accumulation to non-alcoholic steatohepatitis (NASH), and cirrhosis (1). While the pathogenesis of NAFLD is unclear, the two-hit hypothesis was an established theory that encompasses: “first-hit” being a sedentary lifestyle, high fat diets, or insulin resistance followed by the “second-hit” a cascade of inflammation by engorged adipocytes and hepatocytes (1,3-5). Phospholipids differ by their polar groups and hydrophobic hydrocarbons fatty acids. In particular, the lipid composition of these phospholipids leads to the creation of different microenvironments that can account for protein enrichment or dispersion (6). These membrane properties are changed by the phospholipid’s chain length and number of double bonds of incorporated fatty acids (6). Studying hepatic liver fatty compositions in humans would be quite invasive, so the use of plasma phospholipids has been suggested as biomarker to determine any changes in phospholipid metabolism in the liver (7,8). The first study to find a connection in individuals with NAFLD and NASH found the major circulating fatty acids in phosphatidylcholine (PC), phosphatidylethanolamine (PE) were palmitic acid (PA),

stearic acid (SA), and oleic acid (OA) (7). Stearic acid was increased in plasma PC in both NAFLD and NASH compared to controls (7). A study in 2016 comparing healthy controls to individuals with simple steatosis (SS) and NASH, found that AA and docosahexaenoic acid (DHA) was higher in PS in individuals with SS and with NASH (8).

LA is an essential 18 carbon fatty acid that can be obtained from food such as plant-derived oils, and crop seeds (9,10). The prevailing theory is that diets enriched in LA have been associated with inflammation, blood vessel constriction and platelet aggregation due to its conversion to Arachidonic Acid (AA) which is a precursor to pro-inflammatory eicosanoids, however this is not consistently reported (9-11). In healthy adult subjects and others consuming excess LA did not lead to increase inflammation markers or increased AA in serum (9,12). In animal studies, high fat diets enriched in LA negatively correlated with carcass fat, and improved insulin sensitivity, and differentially altered fatty acid composition of triglycerides (TG), Diglycerides (DG) and phospholipids of certain tissues when compared to high fat diets composed of saturated fat (13,14).

**Aims**

1. Compare the effect of two dietary fat sources on fatty acid composition of hepatic phospholipids.

2. Examine if a high fat diet rich in LA reduces liver fat and liver TG.

**Hypothesis**

1. Intake of a high fat diet made with LA rich safflower oil will reduce hepatic fat accumulation and increase incorporation of LA into hepatic phospholipids compared to a high fat diet made with predominately lard in a mouse model of diet induced obesity.

# Chapter 2: Literature Review

## 2.1 Obesity

Obesity has been defined as excess of body fat mass (15). Reliable fat mass quantification requires tools that are not widely available (such as Dual X-ray Absorptiometry, Magnetic Resonance Imaging). As a consequence, obesity has been defined using body mass index (BMI) as a surrogate measurement (15,16). Obesity is classified as having an BMI > 30 kg/m2, with subcategories of Class I-III to assess the degree of obesity (15, 16). Obesity is associated in the pathophysiology of many chronic conditions like insulin resistance, T2DM, dyslipidemia, hypertension, MetS and NAFLD (17).

## 2.12 Adipose Tissue, A Major Endocrine Organ

Adipose tissue has two crucial roles to maintain glucose homeostasis: secretion of appropriate levels of adipokines which can help control behaviors related to feeding, and sequestration of lipids that are related to feeding to avoid increased circulation of free fatty acids and ectopic triglyceride storage (16). Adipocytes can synthesize and release TG, but as their adiposity increases their function as endocrine cells are affected (16). Adipokines such as MCP-1, and TNF-α (Tumor Necrosis Factor-Alpha) modulate an inflammatory response in the cells which in turn attract many macrophages to infiltrate the adipocytes (16,17). This increased TNF-α also attenuates insulin sensitivity and affects the optimal storage of TG (18). Two main sites of fat storage, visceral (VAT) and abdominal subcutaneous (SAT) have been shown to exhibit different metabolic and atherosclerosis profiles (18). Excess VAT in prospective studies have been associated

increased markers of inflammation, oxidative stress, and hepatic steatosis (18). The visceral fat depots release inflammatory cytokines such as TNF-alpha, interlekin-1, and Interlekin-6 (IL-6) enter circulation enhancing NAFLD, pancreatic injury that leads to a decrease in insulin synthesis and secretion (17). Besides the increase of inflammatory cytokines, human subjects have shown increased circulating leptin and decreased circulating adiponectin (19, 20). Leptin is secreted from adipose tissue and is known for its role in controlling energy balance by activating on the brain to decrease food intake, and its role in manipulating glucose and lipid metabolism to maintain energy storage (20). Leptin concentrations will typically decrease during times of starvation, and weight gain causes leptin to increase to limit food intake (21). Human obesity is associated with leptin resistance, and it should be noted that even that leptin is increased in individuals with obesity, in a small fraction of these individuals exhibit low levels of leptin (21). Adiponectin is an adipokine that stimulates fatty acid oxidation in the liver and skeletal muscle (19). A clinical study, comparing control healthy subjects to patients with NAFLD found that decreased circulating adiponectin, was associated with increased insulin resistance (19). In mice, high adiponectin levels have been shown to decrease fatty acid synthesis by reduced expression and activity of FAS (22). It was also shown that adiponectin binding interactions with its receptor increased PPARα activation, which is heavily involved in regulation of fatty acid oxidation (20). Besides its interaction with lipid metabolism, adiponectin may alleviate inflammation by suppression of IL-6 and TNF-α (23).

### 2.13 Obesity, Disrupts Whole Body Glucose Homeostasis

Obesity is a major contributor to metabolic dysfunction of lipids and glucose. Typically, as excessive fat storage occurs, it causes the increase of released excessive fatty acids from increased lipolysis resulting in elevated free fatty acids in the blood that can cause peripheral insulin resistance (16,17). The excessive free fatty acids also increase hepatic glucose production, decrease utilization of glucose by skeletal muscle, and decrease secretion of insulin from pancreatic beta cells resulting in hyperglycemia (17). Insulin resistance typically manifests itself by decreased insulin stimulated glucose transport and metabolism in skeletal muscle and adipocytes with the presence of impaired hepatic glucose suppression in the liver (24). Tissue alterations can occur in obese humans with T2DM such as changes in IRS-1 expression, the insulin receptor needed for the cascade of insulin signaling in adipose (25). However, these alterations in insulin signaling seem to be tissue specific, as in skeletal muscle of T2DM subjects that have altered PI3CK signaling but normal expression of IRS-1 (25). Another unknown is of insulin resistance's interrelationship of circulating insulin, secretion of insulin and clearance. A recent study found that plasma insulin and insulin secretion were increased in obese individuals but decreases in insulin clearance were only found in the subset that were considered insulin resistant (24). Liver is one of the major tissues need for proper insulin clearance as contributes around ~50% of total insulin removal (26). In a clinical study with human patients that were classified according to a low fat or high fat liver content, found that reduced insulin clearance was inversely associated with liver fat content (27).

## 2.2 Non-Alcoholic Fatty Liver Disease

NAFLD can be defined as hepatic fat accumulation in the liver in patients who do not consume excessive alcohol (1,28). Risk factors for NAFLD included obesity, hyperglycemia, T2DM, and insulin resistance (1,28,29). The prevalence of this condition affects about 30% of adults in the general population and about 10% of obese children (30-32). NAFLD encompasses a spectrum of liver conditions and this includes hepatic steatosis, nonalcoholic steatohepatitis, fibrosis, and cirrhosis of the liver (29,32,33). Clinically, hepatic steatosis has been defined as hepatic TG levels as >55 mg per lipid, or the presence of >5% TG cytosolic lipid droplets of hepatocytes (28,33). Hepatic free fatty acid sources for the increased TG accumulation include dietary TG from chylomicrons, de novo lipogenesis in the liver, lipolysis from adipose tissue, diminished export of lipids out of the liver, reduced beta oxidation and increased reactive oxygen species (1,29). However, the literature suggests that 60-80% of liver fat comes from plasma FFA, which is theorized to come from the insulin resistant adipose tissue causing increased lipolysis (34). De novo lipogenesis is only thought to contribute around 5-25% of TG (34,35). NASH is defined by the injury of hepatocyte injury including ballooning, inflammation, cell death, and collagen deposition (fibrosis) (33). Currently this promotion of transition steatosis to steatohepatitis and cirrhosis have not been clearly defined but theorized by the ‘two hits hypothesis’ (1,28,35). The ‘two hit hypothesis’ is described as the first hit of lifestyle like sedentary, high fat diet, and insulin resistance can act as the first hit, as the

second hit involves inflammatory cascades, oxidative stress and fibrogenesis leading to NASH (35).

**2.21 Mitochondrial Dysfunction and NAFLD**

In recent literature suggest that NAFLD, may be a part of both hits in this theory since mitochondria are involved in both beta oxidation, and cellular source of reactive oxygen species in rodent models as well as humans (29,37-40). It is the synchronized induction of pathways involved in lipid accretion, oxidation, and gluconeogenesis that causes oxidative stress, and dysregulated glucose control (39). In the start of the disease, an upregulation of PPARα promotes increased fatty acid delivery to mitochondria from CPT-1 (40). This is theorized to be an attempt by the mitochondria, to stop lipid accumulation from occurring in the hepatocytes. Studies using a choline deficient mouse model found that the mice had an increase in OXPHOS efficiency, but several weeks later the mitochondria exhibited alterations in the ETC complexes, and reduced ATP synthesis (47). In humans, increased liver TG content was positively associated with hepatic TCA flux, suggesting that oxidative metabolism is still functioning (39). From the increased generation of ROS, mitochondria DNA is quite susceptible due to its location within the mitochondria. NAFLD has increased biomarkers of oxidized mitochondria DNA, and altered organelle biogenesis such as PGC-1α, NRF-2 and TFAM (42,43). PGC-1α is a downstream target of glucagon, increased cAMP that is important regulator of gluconeogenic enzymes, and fatty acid oxidation in fasted livers, and diabetic livers (46). A clinical study discovered that individuals with higher triglyceride accumulation had increased endogenous glucose production compared to low triglyceride

accumulation (39). The subjects with increased triglycerides, also had an increase anaplerotic flux into mitochondria supporting the increased endogenous production of glucose observed earlier (39). In mice with steatotic livers, there were no significant changes in mRNA of PGC-1α, but they did have a 50% reduction in protein levels, and a 30% decrease in the abundance of NFR-1 and TFAM (43). TNF-α, plays a large role as a marker of systemic inflammation that plays a role as well by changing mitochondrial morphology and function (48). TNF-α induces mtDNA damage, defects oxidative phosphorylation, that leads to increased oxidative stress and less ATP synthesized (49). As all these factors compound upon each other, reduced ATP synthesis leads to cellular apoptosis through the release of cytochrome c due to the mitochondria inability to maintain its structure and function (48).

**2.23 Dietary Fat and NAFLD**

While much of the literature around NAFLD focuses on TG accumulation as the key defect in NAFLD, little is known about fatty acid compositions of lipids in hepatic liver profile. It has been found that diet alters hepatic phospholipids in both animals and human subjects (6,36,50). In a study comparing hepatic fatty acid profiles of mice, with or without NAFLD found altered hepatic fatty acid profiles (36). In NAFLD mice, the fatty acid concentrations of oleic acid (18:1), linoleic acid (18:2), and linolenic acid (18:3) were decreased compared to the control group (36). These mice also had increased concentrations of saturated fatty acids such as myristic (C14:0), palmitic (PA/C16:0), and stearic acid (SA/18:0) (36). Increased dietary fat intake was also shown to lower hepatic gene expression involving metabolism of n-3 and n-6 polyunsaturated fats (PUFAS) (42).

PUFAs can modulate lipogenesis, inflammation, and metabolism of lipids through transcription factors (PPARS) (18). Another study comparing stages of NAFLD in humans, found altered oxylipins derived from LA and AA from early stage to the later stage (44). In a cross-sectional study in Canada, which included patients with NAFLD, more than >80% of subjects from each group (healthy vs simple steatosis vs NASH) did not consume enough linolenic acid or linoleic acid (45). Moreover, patients with NAFLD or NASH both had increased levels of saturated fats in DAG and TG, and oleic acid besides alter n-6 metabolism, upregulation in oxylipin production might suggest that patients with NAFLD may have increased PUFA requirements.

**2.3 Linoleic Acid**

Linoleic Acid is an eighteen-carbon essential omega-6 fatty acid. Traditional dietary sources of LA come from crop seeds and vegetable oils such as canola, soybean, sunflower, corn, and safflower (10). Typical dietary intake of LA in the United States is about 6% of energy, and Adequate Intake for women and men between the ages of 19 – 50 years old is 12-18 grams/day (51). In the United states, soybean oil currently accounts for 45% of dietary LA (51). LA has been labeled in research as a pro-inflammatory fat, since endogenously LA can be converted to AA which are substrates for the formation of pro-inflammatory prostaglandins (PGs) such as PGE2, leukotrieneB4, thromboxane A2 from the actions of cyclooxygenases (9,51,52). The biggest concern is the increase of the pro-inflammatory PGs may increase other markers of inflammation such as TNF-α, C-reactive protein (CRP), and IL-6 (9). These results have not been consistently reported, in fact in epidemiological studies increased LA led to reduced cardiovascular disease and

showed a negative or null relationship with inflammatory markers (11). In healthy adults increased LA content was not found to increase serum AA or in erythrocytes (9). Also, in tracer kinetic studies the fractional conversion of LA to AA is believed to be between 0.3% or 0.6% (51). LA has also been discovered to be a natural ligand for peroxisome proliferator-activated receptors (PPARS), nuclear receptors involved in regulation of lipid metabolism, energy balance, inflammation, and atherosclerosis (53,54). The three discovered isoforms of PPARα, PPARβ/δ, and PPARγ have been linked to obesity, diabetes, dyslipidemia, and inflammation (55,56). PPARα is responsible for liver, and skeletal muscle lipid oxidation as well as glucose homeostasis, through the regulation of genes involved in mitochondrial beta-oxidation, fatty acid uptake and TG catabolism (55). PPARβ/δ is expressed in hepatocytes like the Kupffer cells and hepatic stellate cells, with effects related to fatty acid and glucose metabolism (55). PPARβ/δ activation in Kupffer cells has also been associated with anti-inflammatory effects by attenuation of IL-6 induced inflammation (57). PPARγ is more highly expressed in adipose tissue and plays role in adipose differentiation and increasing the expression of important markers involved in lipid metabolism (57,58). PPARγ regulates expression of fatty acid transporter protein 1 and CD-36, both needed for lipid uptake (57). Increased PPAR1γ (liver isoform) expression is a trait of fatty livers, and this increased expression activates lipogenic genes and increases de novo lipogenesis in the liver (57,58).

### 2.32 Linoleic Acid, Obesity, and Biomarkers of Metabolic Syndrome

The inclusion of LA and how much to consume in the diet has been highly debated in the current literature regarding effects on obesity and related diseases (59-62). A meta-

analysis comparing the effects of carbohydrates, PUFAs, and monounsaturated fatty acids (MUFAS) on glucose homeostasis found that replacing carbohydrates with PUFA significantly lowered hemoglobin A1c (HbA1c), and fasting insulin (60). Replacement of saturated fats by PUFAs significantly lowered glucose, HbA1c, C-peptide and HOMA-IR scores (60). It should be noted, that PUFAS also contain omega-3 fatty acids, so these benefits may not be completely contributed to just LA, or other omega-6 fatty acids. However, from clinical studies in literature and in our own lab have corroborated some similar findings. A past clinical study completed in the Belury lab, found that erythrocyte linoleic acid, was associated with improvements in body composition by increased lean mass compared to trunk adipose mass (63). Another past study completed in the Belury lab showed that post-menopausal women consuming 8 grams of LA-rich safflower oil had decrease CRP, HbA1c and increased HDL cholesterol after 16 weeks (64). A study in Japanese adults found that increased serum linoleic acid was associated with decreased visceral fat, fasting glucose, HbA1c and systolic blood pressure (65). The subjects with diabetes in the study also had lower serum linoleic acid compared to the normal subjects (65). In the absence of weight loss, in abdominally obese patients consuming an isocaloric diet high in PUFAs (sunflower oil, 50% LA) reduced liver fat, and led to moderate improvements in blood lipids with no adverse effects on oxidative stress and inflammation compared to those consuming a diet high in saturated fat (66). Though as short-term study (10 weeks), its findings reported improvements that were also found in a study ran in our lab with post-menopausal women consuming safflower oil (64). In an overfeeding study, comparing muffins baked with either sunflower oil (LA) or palm oil

(SFA) found that the subjects consuming the muffins baked with sunflower oil after seven weeks had increased lean tissue, and prevented deposition of liver, visceral and total fat compared to those consuming saturated fat rich muffins despite similar weight gain (67).

**2.4 Cardiolipin**

Cardiolipin (CL) is a unique phospholipid dimer that contains four fatty acids, that is found exclusively in the mitochondria (68-78, 80-83). CL is mainly localized to the inner mitochondrial membrane, where it typically constitutes around 20% of the total mass of phospholipids in the mitochondria and represents about ~15% of the mitochondrial phospholipids (73,75). CL has several key functions vital to the mitochondria that include anchoring of mitochondrial proteins, release of cytochrome c in apoptosis, facilitating proton leakage, along with being a structural components for membrane fusion, and mitochondrial architecture (68-70, 72). The fatty acid composition of CL is typically dominated by the incorporation of four LA side chains in metabolically active tissues such as cardiac and skeletal muscles, and liver tissues (69,71). A unique feature of CL is that dominate species of CL have symmetrical acyl chains where both sn-1 and sn-2 positions are identical fatty acids, and this property is conserved across mammalian species (69). While total CL content varies from 5-20% of mitochondrial membranes in mitochondrial dense tissues, the dominant species of CL is the $LA_4CL$, which consists of 55-80% of total CL species (75, 76). $LA_4CL$ is not derived directly from the de novo biosynthesis of CL but by a remodeling process that occurs with the help of phospholipases and acyltransferases such as taffazin (TAZ), ALCAT and MLCLAT (71).

It has been theorized that pathological remodeling of cardiolipin is related to mitochondrial dysfunction including diabetes, obesity, heart failure, hepatic steatosis, and Barth Syndrome (70-73).

**2.41 Biosynthesis of Cardiolipin**

The enzymes involved in the biosynthesis of CL besides cardiolipin synthase, display limited acyl specificity (74,76). The final form of CL is not from biosynthesis but by the remodeling of the acyl side chains in the inner mitochondrial membrane (IMM) (68,71,74,76). The pathway begins in the outer membrane with the addition of an acyl-CoA to glycerol 3 phosphate (G3P) to form lysophosphatidic acid (LPA) from glycerol-3-aycltransferase (GPAT), which uses saturated fatty acids as substrates (68, 77). LPA is then converted to phosphatic acid (PA), and then is transported into the IMM to be synthesized into CDP-DAG by CDS (68,78). Another mitochondrial assembly protein known as Tam41 also has a CDP-DAG synthase function to convert PA to CDP-DAG. In a study with yeast, cells lacking Tam41 had increased accumulation of PA, and decreased CL production (79,80). The rate-limiting step is the conversion of CDP-DAG to glycerol phosphate (PGP), which is then hydrolyzed by protein tyrosine phosphatase mitochondrion 1 (PTPMT1) to form phosphatidylglycerol (PG) (68,81, 82). PTPMT1 is localized to the IMM, and whole-body deletion in mice leads to embryonic lethality, whereas lipid analysis from deficient fibroblasts shown increased accumulation of PGP and decrease in PG (82). The final step in is the synthesis of cardiolipin is the addition of another addition of a phosphatidyl group from CPD-DAG, to form nascent cardiolipin by an enzyme called cardiolipin synthase (CLS) (83). CLS exhibits acyl substrate specificity

and here it is thought that remodeling takes place with the help of phospholipase-A2 to deacylated nascent CL to monolysocardiolipin (MLCL) (77, 84). MLCL can be resynthesized to CL by three remodeling enzymes: tafazzin, LMCL AT-1 and ALCAT-1 (68,71,73,74).

**2.42 Remodeling Enzymes of Cardiolipin**

The remodeling enzymes TAZ and ALCAT-1 show limited acyl specificity, but as mentioned TAZ activity can be modulated by the physical properties of the membrane itself (74,80,85). TAZ is membrane protein that resides on the outer part of the IMM, and on the inner face of the OM (86,87). In vitro studies have shown that TAZ exhibits significant transacylation activity with non-bilayer forming phospholipids such as PE & CL, and from divalent cation stimulation TAZ has a strong preference for LA compared to Oleic acid (OA) (88). Other lipid packing studies suggest microdomains in the mitochondria that are rich in CL and PE help form non-bilayer membranes, relevant for curvature and membrane fusion in mitochondria (70, 89). From studies in isolated mitochondria, it also suggested that virtually all the CL present in mitochondria is associated with proteins (90,91). In support of this CL microdomains are only present in areas of high curvature, and maybe associated with increased super complex association in the IMM (70,89). Microdomains of CL may be forcing acyl specificity on TAZ by negative curvature formation, a common feature of non-bilayer phospholipids such as CL and PE (89). The microdomains of CL, perhaps in the cristae, may hold the mitochondrial acyl transformation mechanisms where TAZ can transfer acyl substrates from any phospholipid that is forced to interact with an CL, and LA rich regions which can force

substrate specificity. Unlike TAZ, ALCAT1 has a selective preference to add substrates to lysocardiolipin. It has also been linked to the progression of insulin resistance, NAFLD, and type two diabetes by abnormal cardiolipin remodeling (71,74,89,92,93). ALCAT1 is located on the mitochondrial associated membrane (MAM), a spot that is the crossroads for phospholipid synthesis between the endoplasmic reticulum and mitochondria (71,92,93). ALCAT1 activity associated in the mitochondria displayed highest activity towards stearoyl-CoA, a saturated 18 carbon fatty acid, along Oleoyl-CoA and Linoeyl-CoA (100). In ALCAT1 KO mice the onset of NAFLD was prevented. ALCAT deficiency also restored mitochondria turnover, architecture, and oxidative phosphorylation (92). Unlike TAZ and ALCAT1, MLCLAT is the only enzyme that has an acyl specificity for LA (71,94). In purified enzyme studies, MLCLAT exclusively used MLCL and no other lysophospholipids, and utilized unsaturated acyl-CoAs (oleoyl & linoleoyl) compared to saturated acyl-CoA (94).

**2.43 Cardiolipin and ATP Synthesis**

The primary function of the mitochondria is to generate ATP by oxidative phosphorylation (95). Although CL has been shown to interact with multiple IMM proteins, metabolites, and carriers that are exclusively associated with the IMM, its main function is ATP synthesis (95,96). Cardiolipin is required for optimal function of complex I, complex III, complex IV, and ATP synthase, ADP/ATP carrier (68,74, 83,95-97). In yeast and bovine hearts, respiratory complexes to assemble as functional units known as super complexes (96, 98). In mammalian mitochondria, complex I was highly associated to complex III, and small amounts of complex IV (98). In yeast, the lack of CL

causes functional disruption of ATP synthesis along with respiratory super complex formation. Currently in literature, the importance of CL in the formation of supercomplexes in mammalian mitochondria has not been discerned but aberrant respiratory super complex formation has been observed in fibroblasts from Barth syndrome patients (98,99). The abnormal CL acyl profile is a hallmark of Barth Syndrome and has, been associated with increased reactive oxygen species, and reduced oxidative efficiency (85). In mammalian mitochondria, there are several proposed models of super complexes formation, including the current existence of at least two, one involving: Complex I, III, IV and the other consisting of complexes III and IV (95, 98). Along its role in the formation of super complexes, CL has a unique function as a proton trap. Thomas Haines and other researchers theorized the importance of anionic phospholipids to conduct protons not through phospholipids but on the outside near the charged head groups (101). Given the pH of the IMM, is around 8.0, which is slightly above neutral, as CL at this point has one negative charge which is perfect for grabbing protons (102). As mentioned earlier, the theorized existence of microdomains of CL, or patches of dense CL would be perfect to keep protons near the membrane for optimal cycling of protons through the oxidative complexes, and ATP synthase (102).

**2.5 Phospholipids**

Phospholipid membranes are vital structural components of all cells. The cellular or plasma membrane protect the cell from external influences, organizes cells into distinct compartments and help determine the flux of components into and out of cells (103). Phospholipids, such as phosphatidylcholine (PC) and phosphatidylethanolamine (PE) are

the two most abundant eukaryotic membrane lipids of the plasma membrane, that consist of a polar head group, glycerol backbone, with a two fatty acid tail (6,103,105-108). These fatty acids tails can be categorized into saturated, monounsaturated, and polyunsaturated with further divisions such as omega-3 (n-3) and omega-6 (n-6) (108). It has been well reported that both the chain length and the number of double bonds of these fatty acids have major influences on the physical properties of phospholipids including permeability and fluidity (108). Besides PC and PE other structural phospholipids include phosphatidylinositol (PI), phosphatidylserine (PS), PA and CL. It has been noted that while organelles and their membranes contain all these phospholipids (except CL), they are distributed unequally among the bilayer membranes (106). For example, the synthesis of CL is exclusive to the mitochondria IMM whereas PE is not a mitochondrial exclusive phospholipid due to the multiple pathways to synthesize PE (105-107). The formation of PC and PE rich membrane induce specific physical properties of their membranes, with PC as bilayer forming and PE as a non-bilayer forming membranes (104,109,111). PC is the major bilayer forming phospholipid, as it has a cylindrical geometry with the fatty acid tails facing each other and head groups that interact with aqueous phase (108,109). Non-bilayers phospholipids have a small head group, and bulky fatty acid side chains which results in the formation of a conical shape (104,110). PE and CL are the two major non-bilayer phospholipids which are both able to synthesize in the mitochondria compared to the other phospholipids that are synthesized in the endoplasmic reticulum (106,109-111). In vivo, both PE and CL are present in mitochondrial membranes where they induce curvature into mitochondrial membranes, and are important for

mitochondrial fusion, mitochondrial contact sites of the endoplasmic reticulum (ER) and the movement of proteins (109). In areas of high curvature ranging from the mitochondrial cristae to *Escherichia Coli* membranes the formation of CL and PE microdomains may exist and lead to enrichment of these two phospholipids (105,109).

### 2.51 Synthesis of Phospholipids

The ER is the main organelle for phospholipid production and PA is vital to the synthesis of all the other phospholipids (103,106,109-111). From PA, DAG or CDP-DAG can be formed and both are needed for mammalian synthesis of the major phospholipid species (106,107,109,110).

### 2.52 Phosphatidylcholine

Choline is an essential amine for mammals, and the main fate of choline is in the synthesis of PC via the Kennedy Pathway, which can account for about 95% of the total choline pool in most tissues (106,107,112). PC is the most abundant phospholipid in organelles and accounts for about 40-50% of the total phospholipids mainly in the outer leaflet of the phospholipid bilayer of organelle membranes (106). Synthesis of PC through the Kennedy pathway involves the reaction of three key enzymes: choline kinase (CK), phosphocholine cytidylyltransferase (CCT), and choline phosphotransferase (CPT) (106,107). First, choline is imported into the cells where it is phosphorylated to phosphocholine by CK (106,107). Then phosphocholine is transformed into CDP-choline by CCT, which is the rate limiting step of the pathway (106,107,112,113). The final step of the pathway is on the ER membrane, and CCT transfers a DAG group to CDP-choline forming PC (106,107,113). An alternative pathway to produce PC is through the PE-

methyl transferase 1 (PEMT1), which is also localized to the membrane of the ER (1,106,107,113,114). Currently, evidence suggests that only hepatocytes utilize this pathway to produce PC generating around 20-40% of liver PC (107,113). The PEMT utilizes S-adenosylmethionine (SAM) as the methyl group donor for the conversion of PE to PC (1,107,113-115). In PEMT1 KO mice little changes in hepatic phospholipid composition were noted, and it was found that these mice had increased CPT activity to compensate for the lack of PEMT (115). Maintaining PC homeostasis seems very important, as it has been calculated that the mouse liver secretes its entire PC pool to produce lipoproteins and bile (107). It has been theorized that the liver is able to compensate to ensure a component source of PC is generated in the liver upon inhibition of PEMT or the CDP-choline (Kennedy Pathway). A third way to generate PC is by Land's Cycle, with LPCAT (lyso-phosphocholine acyltransferase) producing PC from LPC and acyl chains (1, 116,117).

**2.53 Phosphatidylethanolamine**

PE can be synthesized from three major pathways: Kennedy pathway, PS decarboxylase (PSD1) or by the Land's Cycle (1,106,107, 109, 110, 112,113). Synthesis of PE from the Kennedy pathway requires a set of enzymes instead of using choline the enzymes utilize ethanolamine and are appropriately named ethanolamine kinase (EK), phosphoethanolamine cytidylyltransferase (ECT), and CDP-ethanolamine phosphotransferase (EPT) (105,106). Just like PC, the rate limiting enzyme of this pathway is ECT and the final enzyme is located on the ER, which converts CDP-ethanolamine:1,2 diacylglycerol and diacylglycerol to PE (1,106,107). The second

pathway to synthesize PE utilizes the enzyme PSD1 which is, located in the inner mitochondrial membrane (1,106,107,109,110). This enzyme uses extramitochondrial PS formed on ER MAM regions and then it is imported into the mitochondria (106,107,109). While it is unclear how much PE is quantitatively produced from each pathway, KO mice of both PSD1 (PS decarboxylation) and ECT (Kennedy Pathway) are embryonic lethal regardless of the presence of the other functional pathway (117,118). These two studies indicate that these two separate pools of PE produced in the ER and mitochondria may be functionally distinct. In another study, PSD1 KO mice had altered mitochondrial structures, with decreased respiratory capacity, ATP production and super complex formation between complex I and IV (120). It is theorized that the high membrane curvature at cristae tips may impose certain constraints on the IMM that leads to the enrichment of non-bilayer phospholipids such as PE, and CL (109). Disruption of PE has been showed to lead to impairment gradient, reduced protein import into the mitochondria, but with notable increases in super complex stabilization (110). As PE is the second most abundant phospholipid in mammalian cells and the mitochondria, particularly the IMM is composed of 30-40% of PE (106). The third final pathway is a way to recycle PE, through the Land's Cycle with the use of Acyl-Co: lysophospholipid acyltransferases using a LPE with the addition of a fatty acid (1,116,117).

**2.54 Phosphatidylinositol**

The mammalian synthesis of PI is from the enzyme Phosphatidylinositol synthase (PIS), in catalyzing myo-inositol and CDP-DAG to form PI and CMP in the presence of magnesium (121,122). PIS is on located on the ER membrane, and has been isolated from

the plasma membrane, however it is unclear which one is more active within cells (106,122). PI is a minor and essential membrane component which constitutes somewhere between 10-15% of total phospholipids in cells (106). PI is involved in protein membrane anchoring, and is the precursor for secondary messengers such as inositol-polyphosphate and DAG (122). PI is unique in the fact that the inositol head group can be phosphorylated multiple times, and these polyphosphates are important for $Ca^{2+}$ and PKC signaling pathways (123). Just like the other PLs, PI can be synthesized from Lyso-PI from the Land's Cycle (116).

**2.55 Phosphatidylserine**

The mammalian synthesis of PS is in the MAM (mitochondrial associated membrane) region of the ER, with the two enzymes: phosphatidylserine synthase 1 (PSS1) and PSS2 (124). The synthases catalyze phospholipid head group exchanges between PC or PE and replaces the head group with a serine (103,106,124). Typically, the PS, synthesized in the ER is transported into other organelles, especially the mitochondria as mentioned earlier as one of the key functions is the synthesis of PE (1,106,107,109,110,124). The supply of PS transported to the mitochondria is essential for PE formation, and it notable that PS content in mitochondria is very low, around 1-5% of total phospholipids in mitochondria (103).

**2.56 Cardiolipin**

See cardiolipin Section 2.41 for the synthesis and remodeling of cardiolipin.

# Chapter 3: Study Design and Methodology

Hypothesis: A LA-rich diet will increase the amount of LA incorporated into hepatic phospholipids and reduce hepatic fat accumulation. To test this hypothesis the fatty acid composition of different phospholipids will be analyzed in mice consuming a high fat diet made with LA-rich safflower oil or a high fat diet made with lard.

## 3.1 2020 HF LA Study

## 3.2 Animals, diets, and experimental design

Twenty-four 9-weeks old male C57BL/J6 mice (N=24) were obtained from Jackson Laboratory (Bar Harbor, MA, USA). Mice arrived in groups of (3-5) and were then moved into individual housing on Day -11 in a vivarium with room temperature of 22 ± 0.5°C and on a 12-hour light/dark cycle, with free access to food and water. The mice were able to acclimate to their new environment for two weeks and cages were equipped with enrichment objects. After the acclimation period, mice were randomized into two diet groups. The lard (LD) diet was a semi-purified diet with the fat source from lard (D20012819, Research Diets Inc. New Brunswick, NJ, USA), while the safflower oil (SO) diet (n=12) was an identical diet formula with the exception of the fat source which was high LA safflower oil (D19081203B, Research Diets Inc., New Brunswick, NJ, USA; Table 4.1). Body weight and food intake was measured every two days.

Mice were euthanized by cervical dislocation in pairs for the mitochondrial function assay starting on day 101. The mice not used for mitochondrial assays were

anesthetized under isoflurane for blood collection via cardiac puncture and before euthanization via cervical dislocation. Blood was collected by cardiac puncture, deposited into an EDTA-coated collection tubes, and stored on ice until plasma was prepared. Tissues were collected, and immediately flash frozen in liquid nitrogen. Frozen tissues were weighed later and stored at -80°C until further analysis, as described below. All procedures were approved by The Ohio State University Institutional Animal Care and Use Committee.

### 3.3 EchoMRI for Body Composition

To assess time-course changes in body composition of live mice, EchoMRI (Houston, TX, USA) was used at three timepoints: Day 0, Day 65 and Day 100.

### 3.4 Fasting Glucose

Mice were moved to clean cages without food and bedding and transferred to a quiet room for 6 hours prior. Blood glucose was collected by pricking the tip of the tail with a 20-gauge needle. Blood glucose was monitored using the OneTouch Ultra glucometer (Lifescan, Inc. Milpitas, CA, USA) on Day -1, 79, 85, 91.

### 3.5 Insulin-stimulated glucose uptake

An insulin tolerance test was used to assess insulin sensitivity. Mice were moved to clean cages without food and bedding to a quiet room for 6 hours prior to the start of the insulin tolerance test (ITT). Blood glucose was collected by pricking the tip of the tail with a 20-gauge needle and blood glucose was measured using the OneTouch Ultra glucometer (Lifescan, Inc., Milpitas, CA, USA). After measuring baseline(fasting) blood glucose,

mice were given a bolus of 0.75 U/kg insulin by intraperitoneal injection. Blood glucose was measured at 0,15, 30, 45, 60, 90, and 120-minutes post-insulin injection.

### 3.6 Glucose Clearance

A glucose tolerance test was used to assess if diet affected glucose clearance in the mice at Day 91. Mice were moved to clean cages without food and bedding and transferred to a quiet room for 6 hours prior to the start of the glucose tolerance test (GTT). Blood glucose was collected by pricking the tip of the tail with a 20-gauge needle. Blood glucose was monitored using the OneTouch Ultra glucometer (Lifescan, Inc., Milpitas, CA, USA). After measuring baseline (fasting) blood glucose, mice were given an intraperitoneal injection of glucose (2 g/kg). Blood glucose was then measure at 0,15, 30, 60, and 120-minutes post-glucose injection.

### 3.7 Grip Strength Testing

Grip strength was used to assess changes in grip force between the diets. The mice were acclimated to forelimb and hindlimb grip strength testing for 1 week prior to the experimental measurements on Day(s) 105, 107 and 109. These practice measurements on the grip strength meter were taken in triplicate, with at least minute of rest between each measurement. Fore limb and hindlimb grip strength were measured on day 113 with the Columbus Instruments Grip Strength Meter (Columbus, OH, USA).

### 3.8 Lipid Analysis

#### Triglyceride and Phospholipid Analysis

Total lipids were extracted from all liver samples with 2:1 (v/v) chloroform: methanol (123). For analysis of total lipids and TG, the chloroform phase containing the lipids was

transferred to a weighed test tube and dried under nitrogen gas at room temperature. The weight of the empty test tube weight was subtracted from the weight of the dried lipids and test tube to calculate the extracted total lipid. Extraction of TG was obtained using solid-phase extraction (SPE) (124) and analyzed using an enzymatic colorimetric assay (Millipore Sigma, St. Louis, MO). For analysis of the phospholipid extraction, (phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, phosphatidylglycerol and cardiolipin) were extracted and separated from the total lipid sample (SPE) according to Suzuki et. al (125) with cartridges from Agilent Technologies, Inc. (Folsom, CA). For fatty acid analysis, fatty acid methyl esters for each phospholipid and for the diets were prepared using 5% hydrochloric acid in methanol (126) at 76°C overnight. Fatty acid methyl esters were extracted with hexane and analyzed via gas chromatography on a 30-m Omegawax ™ 320 fused silica capillary column (Supelco, Bellefonte, PA) using helium carrier gas. Oven temperature started at 175°C and increased at a rate of 3°C/min until reaching 220°C. Retention times of samples were compared to standards for fatty acid methyl esters (Matreya, LLC, Pleasant Gap, PA, and Nu-Check Prep Inc, Elysian, MN). Fatty acids are reported as percent of total identified (127).

**Cardiolipin Analysis**

CL was quantified using previously published methods by our collaborator, Dr. Genevieve Spargagna (University of Colorado), with normal phase liquid chromatography coupled to electrospray ionization mass spectrometry in an API 4000 mass spectrometer (Sciex, Framingham, MA, USA) (128). Liver mitochondria samples

were homogenized using a glass-on-glass homogenizer in PBS and protein was quantified from the homogenate (Bradford plus; Thermofisher Scientific, Waltham, MA, USA). Lipids were extracted by the modified Bligh Dyer method according to previously published methods with 1000 nmol tetramyristal CL as an internal standard (Avanti Polar Lipids, Alabaster, AL, US) (129).

### 3.9 Statistical Analysis

Data are represented as the mean ± the standard error of the mean (SEM) or standard deviation (SD). Differences between LD and SO groups were analyzed by two sample t-tests. Statistical outliers were calculated by comparing individual values to a cutoff range calculated as mean ± 3(SD). All statistical tests were performed using STATA (StataCorp LLC, College Station, TX, USA) and graphed with Graphpad Prism (Graphpad Software, San Diego, CA, USA). All statistical tests were performed at the 5% significance level.

# Chapter 4: Results

**Table 4.1 Diet Composition of the LD and SO diet(s)**

| Ingredient % (grams) | LD | SO |
|---|---|---|
| **Casein** | 200 | 200 |
| **L-Cystine** | 3 | 3 |
| **Corn Starch** | 72.8 | 72.8 |
| **Maltodextrin 10** | 100 | 100 |
| **Sucrose** | 172.8 | 172.8 |
| **Cellulose** | 50 | 50 |
| **Soybean Oil** | 0 | 0 |
| **Lard** | 179.5 | 0 |
| **Safflower Oil, USP** | 23 | 202.5 |
| **Mineral Mix S10026** | 10 | 10 |
| **DiCalcium Phosphate** | 13 | 13 |
| **Calcium Carbonate** | 5.5 | 5.5 |
| **Potassium Citrate, 1 H20** | 16.5 | 16.5 |
| **Vitamin Mix V10001** | 10 | 10 |
| **Choline Bitartrate** | 2 | 2 |
| **Protein** | 24 | 24 |
| **Carbohydrate** | 41 | 41 |
| **Fat** | 24 | 24 |
| **Total (grams)** | 858.15 | 858.15 |
| **Kilocalories** | 4057 | 4057 |

Provided by Research Diets Inc. LD: Lard; SO: Safflower Oil

**Table 4.1 Diet Composition**

Diet composition of the LD and SO diets provided by Research Diet Inc. The data is expressed as (%) of grams.

**Table 4.2 Fatty Acid Profile of Diets**

| Fatty Acid (%) | LD | SO |
|---|---|---|
| **Myristic Acid (C14:0)** | 1.27 +/- 0.026 | 0.204 +/- 0.022 |
| **Palmitic Acid (C16:0)** | 21.7 +/- 0.059 | 7.29 +/- 0.350 |
| **Palmitoleic Acid(C16:1n7)** | 1.63 +/- 0.011 | 0.197 +/- 0.032 |
| **Stearic Acid (C18:0)** | 11.3 +/- 0.076 | 2.66 +/- 0.244 |
| **Oleic Acid (C18:1n9)** | 35.9 +/- 0.093 | 14.5 +/- 0.556 |
| **Linoleic Acid (C18:2n6)** | 26.2 +/- 0.17 | 74.8 +/- 1.25 |
| **Linolenic Acid (C18:3n3)** | 0.926 +/- 0.017 | 0.168 +/- 0.016 |
| **Eicosenoic Acid (C20:1n9)** | 0.548 +/- 0.024 | 0.165 +/- 0.013 |
| **Eicosadienoic Acid (C20:2n6)** | 0.0352 +/- 0.004 | 0.0352 +/- 0.015 |

Analyzed by Gas Chromatography. Data presented as the mean % and +/- SD of total fatty acid profile. LD: Lard, SO: Safflower Oil.

**Table 4.2 Fatty Acid Profile of Diets**

The fatty acid composition of the diets is expressed as percent of total identified and categorized by the diet labels, LD and SO. The diets were analyzed by gas chromatography as described in the methods. The data above are presented as the mean percent ± standard deviation.

### 4.1 Diet Composition & Fatty Acid Profile

The composition of the diets is reported in Table 4.1, and fatty acid composition of the diets is reported in table 4.2. Both diets are isocaloric, with the only difference being the fat source used in each of the diets. The LD is diet is rich in lard, and the SO diet is rich in safflower oil. The major fatty acid species in the LD diet are palmitic acid and oleic acid, while the dominant fatty acid in safflower oil is linoleic acid.

**Figure 4.1 Body Weight**

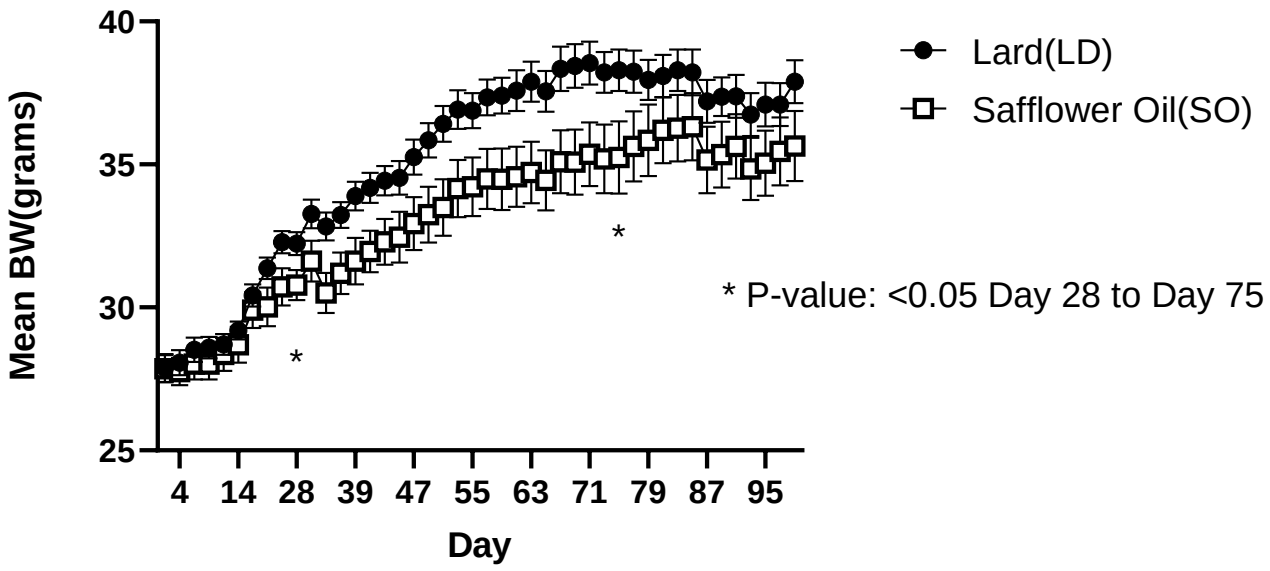


**Figure 4.1 Body Weight**

The mean body weight of each diet until study day 100. N=12 per group. The data above is presented as mean body weight ± SEM in grams. Two sample t-test was used to determine differences between the diets. Data are considered statistically significant at $P<0.05$.

**4.2 Body Weight**

During part of this feeding study, the Covid-19 pandemic required the lockdown of all research labs. In response, we limited person-to-person interactions and were not allowed to work on any experiments that were not considered essential. Therefore, we measured body weights twice per week between day 14 to day 31. From day 14 to day 31, mice were fed once per week but then were changed to feeding every two days from day 35 to the end of the study. Apart from the lockdown for the pandemic, we measured

body weight and food intake every two days. From day 28 to day 75, the mean body weight was significantly different between diets p<0.05. The SO had lower mean body weight compared to the LD diet. The mean body weights were not significantly different after day 75 between diets.

**Figure 4.2 Food Intake**

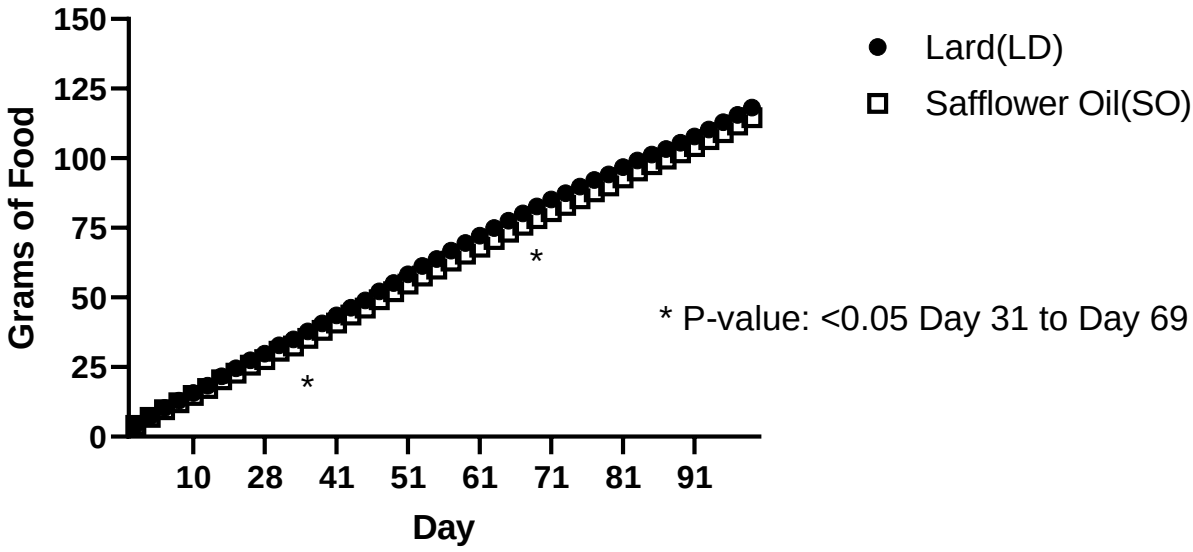


**Figure 4.2 Food Intake**

The mean cumulative food intake until day 100. N=12 for per group. The data above is mean total food intake in grams (SD). Two sample t-test was used to determine differences between the diets. Data are considered statistically significant at P<0.05.

**4.3 Food intake**

To track changes in food intake, we measured food intake every two days. From day 31 to Day 69 the cumulative mean food intake was significantly different between the diets. The SO had consumed less total food during this time, after day 69 the mean total food intake was not significantly different between the two diet groups. This was during the

beginning of the Covid-19 lockdown, and we were attempting to limit our food exchanges and time on campus. After a two-week period of limited food exchanges, we switched back to the two-day food exchanges.

**Figure 4.3 Insulin Tolerance Test**

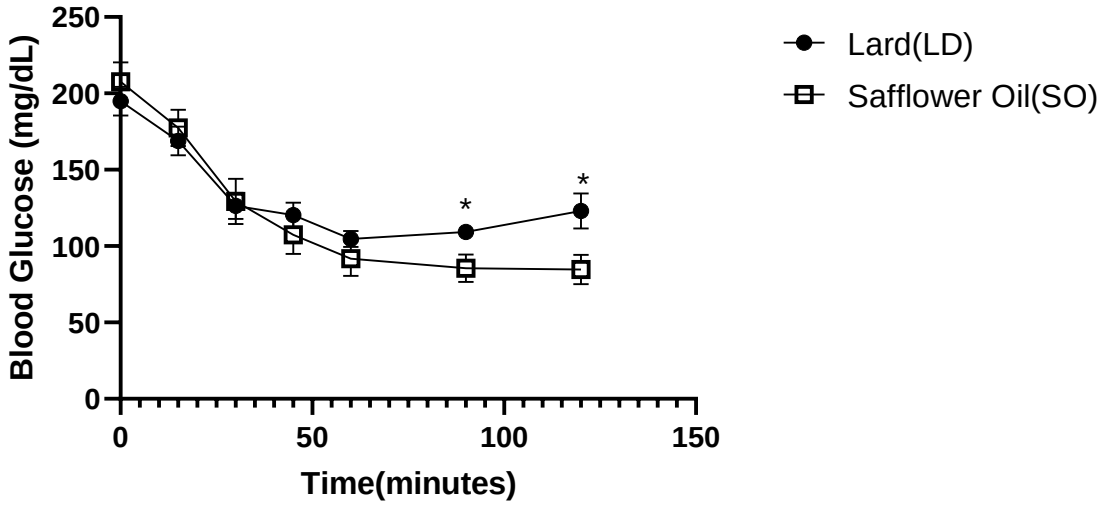


**Figure 4.3 Insulin Tolerance Test**

The change of blood glucose of mice over time. N=12 per group. The data above are presented as the mean blood glucose (mg/dL) ± SEM over time points (0-120 minutes). A two-sample t-test was performed each at time point. Data are considered significantly different at $P<0.05$.

**Figure 4.4 ITT Delta Glucose AUC**

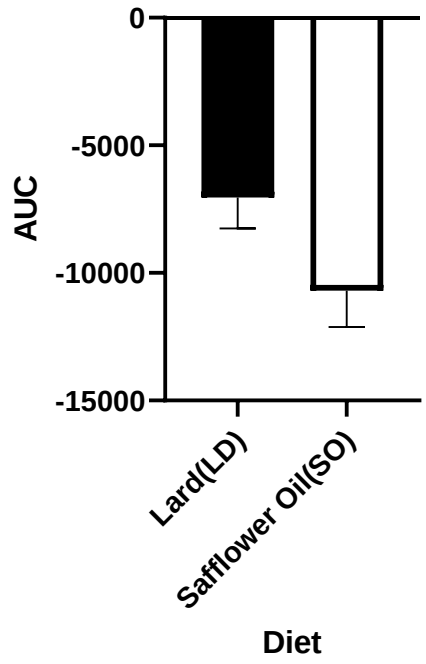


**Figure 4.4 Insulin Tolerance Test**

The area under the curve for delta blood glucose. N=12 per group. Data above is presented as mean area ± SEM. Two sample t-test was used to determine significance between diets. Significance was determined at $P<0.05$.

**4.4 Insulin Tolerance Test**

An insulin tolerance test was used to assess insulin sensitivity. Blood glucose levels before (fasting) and after an intra-peritoneal insulin injection were measured for the next two hours on Day 85. Blood glucose was not significantly different at each of the 15-minute interval time points (0-60 minutes). Blood glucose was significantly different at

the 90-minute timepoint, with mean blood glucose levels of 85.86 ± 9.04 mg/dL, and 109.25 ± 4.89 mg/dL for SO and LD respectively (Figure 4.3, p-value:0.0312). Mean blood glucose was significantly different between the diets at the 120-minute time point, with 84.75 ± 9.68 mg/dL for SO and 120.92 ± 11.46 mg/dL for LD (Figure 4.3, p-value: 0.0241). The AUC is used as indicator of insulin sensitivity (130). The greater the AUC, the lower sensitivity to insulin. AUC was not significantly different between the diets but was trending towards significance with a p-value of 0.06 (Figure 4.4).

**Figure 4.5 Glucose Tolerance Test**

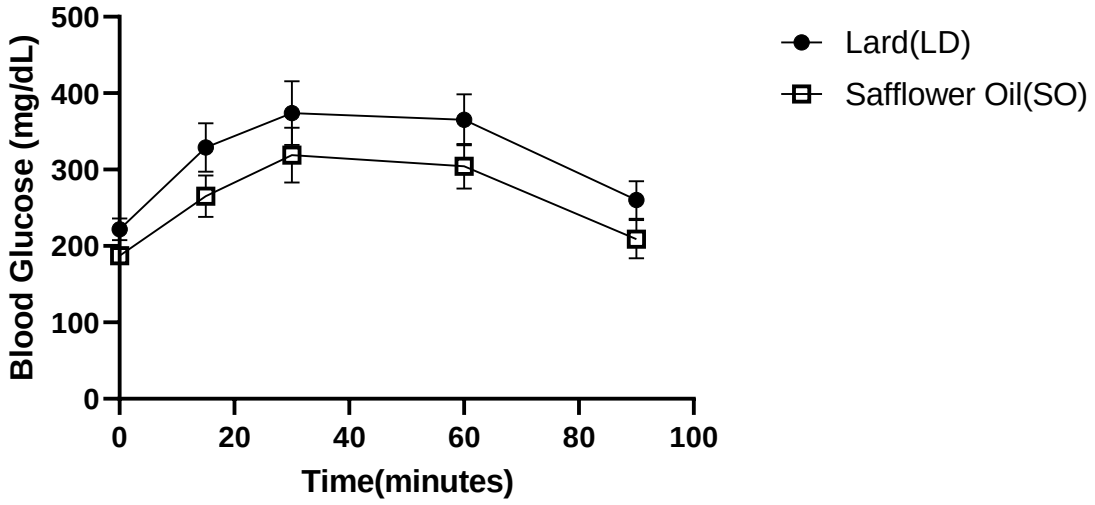


**Figure 4.5 Blood Glucose over Time (GTT)**

The change of blood glucose of mice over time. N=12 for per group. Data above are presented as the mean blood glucose (mg/dL) ± SEM over time points (0-120 minutes). A two-sample t-test was performed each at time point. Data are considered significantly different at $P<0.05$.

**Figure 4.6 GTT Delta Glucose AUC**

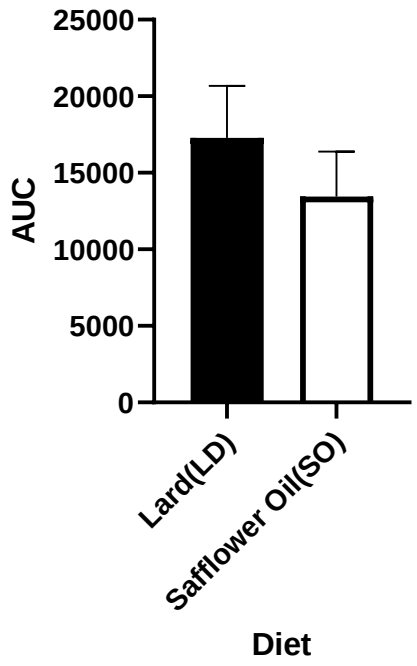


**Figure 4.6 Glucose Tolerance Test**

The area under the curve (AUC) for delta blood glucose. N=12 per group. Data above is presented as mean area ± SEM. Two sample t-test was used to determine significance between diets. Significance was determined at $P<0.05$.

**4.5 Glucose Tolerance Test**

On day 91 the mice were fasted for 6 hours before starting the glucose tolerance test. The mean glucose tolerance was not significantly different between the diets at any of the time points (Figure 4.5). AUC analysis is an index, of whole glucose excursion (131). The delta blood glucose AUC was not significantly different between the diets (Figure 4.6, p-value: 0.2910).

**Figure 4.7 Fore Limb Grip Strength**

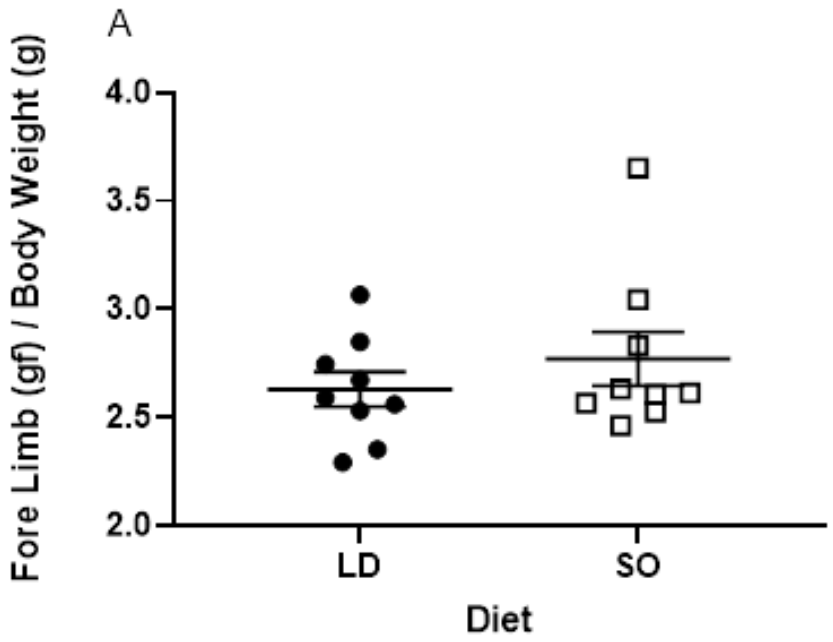


**Figure 4.7 Fore Limb Grip Strength**

Fore limb force of the mice. N=9 per group. The data above are presented the mean ± SEM grams of force. A two-sample t-test was used to determine significance. Significance was determined at p-value: <0.05.

**Figure 4.8 Hind Limb Grip Strength**

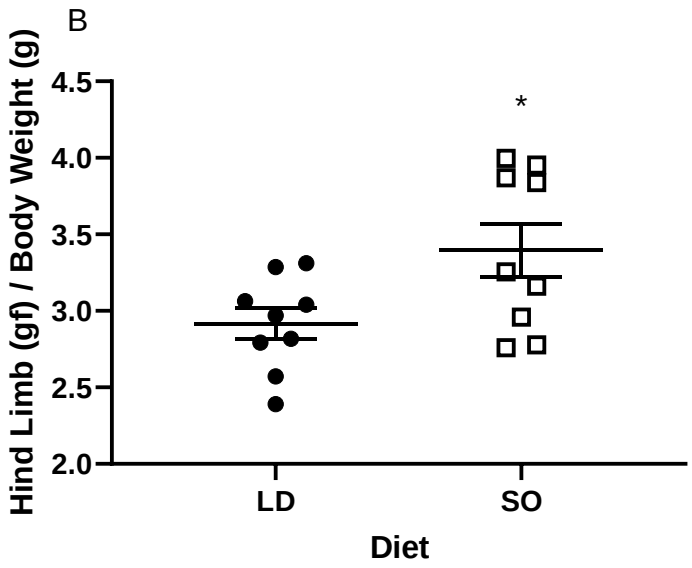


**Figure 4.8 Hind Limb Grip Strength**

Hind limb force of the mice. N=9 per group. The data above is presented as the mean ± SEM of three repeated measurements. A two-sample t-test was used to determine significance. Significance was determined at p-value: <0.05.

**4.6 Grip Strength**

There were no significant differences between the forelimb force between the diets (Figure 4.7). The SO had a significantly higher hind limb force measurements (3.40 ± 0.17) compared to the LD (2.92 ± 0.10) with SO (Figure 4.8, p-value:0.0221).

**Table 4.3 Mean Tissue Mass**

| Tissue | LD (% of BW) | SO (% of BW) | p-value |
|---|---|---|---|
| Heart | .393 +/- .0184 | .410 +/- .0188 | 0.535 |
| Liver | 3.82 +/- .138 | 4.05 +/- .213 | 0.502 |
| Quadriceps | 1.06 +/- .0436 | 1.08 +/- .0517 | 0.721 |
| Gastrocnemius | .790 +/- .0451 | .827 +/- .0459 | 0.576 |
| ING | 3.23 +/- .456 | 2.42 +/- .236 | 0.133 |
| EPI | 5.19 +/- .339 | 5.55 +/- .334 | 0.463 |
| BAT | .511 +/- .0640 | .498 +/- .0572 | 0.881 |

**Table 4.3 Tissue Mass**

Data are presented as mean percent ± SEM of body weight. (N = 7-12) per group. Two sample t-test was used to determine significance between diet. Significance was determined at $P<0.05$.

**4.7 Tissue of Body Mass**

Tissue weights are normalized to body weight since mice were euthanized on different days for mitochondrial function assay. No significant differences in mean % tissue weight were observed between the two diets (table 4.3).

**Figure 4.9 Change in Adiposity (g) Over Time**

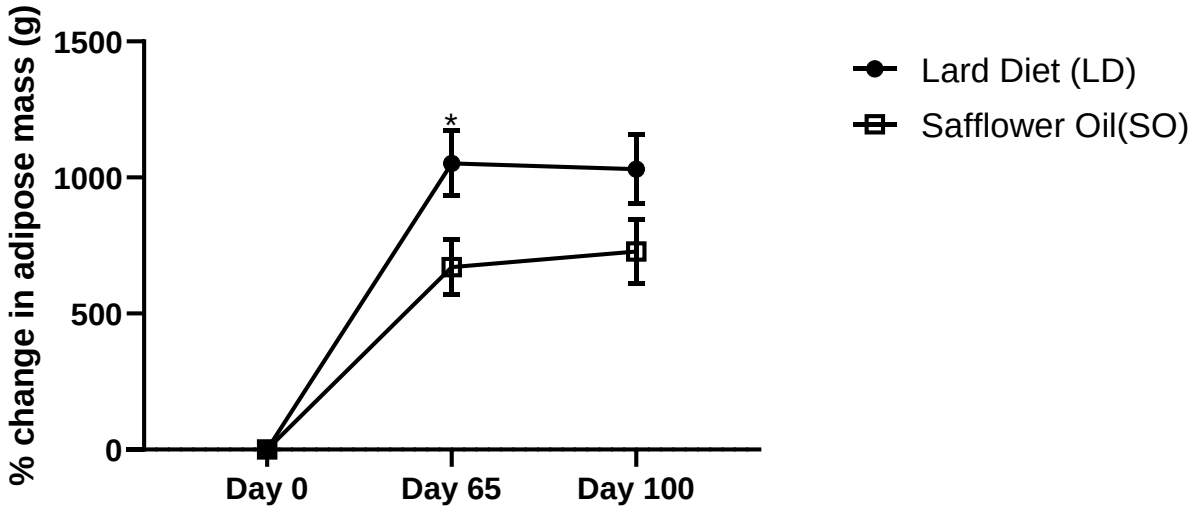


**Figure 4.9 Change in adiposity (g) over Time**

Change in adiposity over the study. N = 12 per group. Data are presented as % change in adiposity (g). A two-sample t-test was used to determine significance between diets. Significance was determined at $P<0.05$.

**Figure 4.10 Change in lean mass (g) over time**

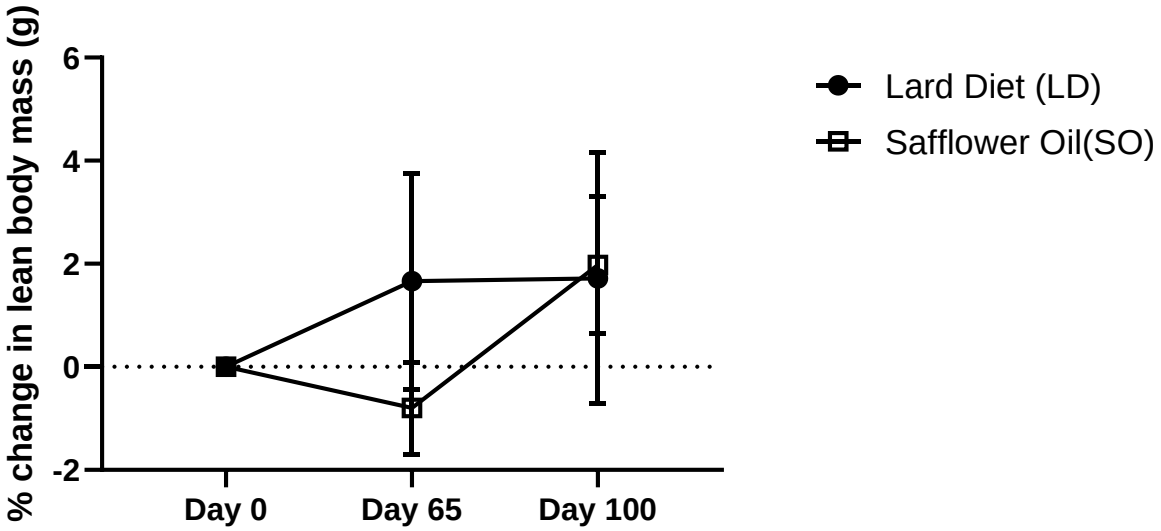


**Figure 4.10 Change in Body Mass (g) over time**

Change in lean mass over the study. N = 12 per group. Data are presented as % change in lean mass (g). A two-sample t-test was used to determine significance between diets. Significance was determined at $P<0.05$.

**4.8 Change in Adiposity & Lean Mass (g)**

To measure changes in lean and adipose mass, echo MRI was used to assess body composition (Figure(s) 4.7 & 4.8). While there was no difference between the two diet groups in the change of adipose tissue mass at day 100, at day 65 the LD diet percent change in adipose mass was larger than the SO diet (p-value: 0.0234). There were no significant differences in percent change of lean mass on either day 65 or 100.

**Figure 4.11 Total Hepatic Lipids (mg/g)**

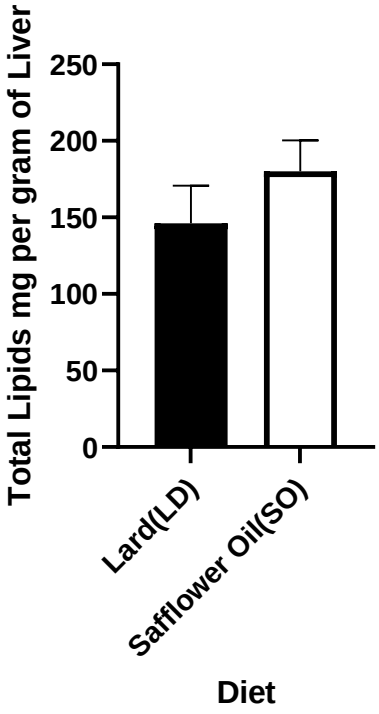


**Figure 4.11 Total Hepatic Lipids (mg/g)**

Total lipid content in the liver. N=12. Data above is presented as the mean ± SEM as mg/g. A two-sample t-test was used to determine significance between diets. Significance was determined at $p<0.05$.

**Figure 4.12 Hepatic Triglycerides (µg/mg)**

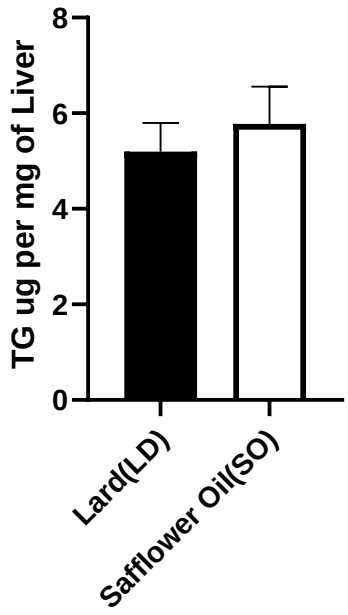


**Figure 4.12 Hepatic Triglycerides (µg/mg)**

Mean triglycerides of the liver. N=12 per group. Data above is presented as the mean ± SEM as µg/mg. A two-sample t-test was to determine significance between diets. Significance was determined at $p<0.05$.

### 4.9 Total Hepatic Lipids & Triglycerides

To determine fat content of the liver, total lipids and TG were measured in the livers. Total lipids includes both non-polar (TG, DAG, sterols) and polar lipids (phospholipids, free fatty acids). There were no significant differences in total lipid content, and mean TG content (p-value: 0.5680) between the two diet groups.

**Table 4.4 Dominant Hepatic Cardiolipin Species by mass to charge (m/z) and likely cardiolipin species**

| m/z | Dominant fatty acid acyl composition | Abbreviation |
|---|---|---|
| 1448 | $(18:2)_4$ CL | $LA_4$ CL |
| 1450 | $(18:2)_3$ $(18:1)_1$ CL | $LA_3OA_1$ CL |
| 1452 | $(18:2)_2$ $(18:1)_2$ CL | $LA_2OA_2$ CL |
| 1474 | $(18:2)_2(18:1)_1(20:4)_1$ CL | $LA_2OA_1AA_1$ CL |
| 1476 | $(18:2)_3$ $(20:4)_1$ CL | $LA_3AA_1$ CL |

**Table 4.5 Percent of Hepatic Cardiolipin Species**

| Species | LD* | SO | p-value |
|---|---|---|---|
| $LA_4$ CL | 29.6 ± 1.99 | 38.0 ± 0.82 | 0.0042 |
| $LA_3OA_1$ CL | 19.5 ± 0.45 | 17.1 ± 0.25 | 0.0013 |
| $LA_2OA_2$ CL | 10.2 ± 0.63 | 6.35 ± 0.53 | 0.0016 |
| $LA_2OA_1AA_1$ CL | 7.95 ± 0.49 | 6.73 ± 0.18 | 0.0771 |
| $LA_3AA_1$ CL | 4.30 ± 0.11 | 6.15 ± 0.14 | <0.0001 |

**Table 4.5 Hepatic Cardiolipin Species**

Percent of dominant hepatic cardiolipin species. N=5 per group. Data is presented as mean percent ± SEM of total cardiolipin species. A two-sample t-test was used to determine significance. Significance was determined at $P<0.05$. *Data presented as mean % ± SEM. N=5. Other cardiolipin species not listed include: 1396, 1398, 1400, 1402, 1422, 1424, 1426, 1428, 1456, 1470, 1472, 1474, 1498, 1500 were not significantly different between diets, and in total contains <25% of total species. Species such as 1404, 1454, 1478, and 1498 were significantly different between diets in total contains <10% of total species.

**4.10 Cardiolipin Speciation**

The dominant hepatic cardiolipin species are listed by mass to charge ratio, fatty acyl composition and by % total (Table 4.4 & 4.5). Values are presented as mean percentage ± SEM. There were significant differences in the hepatic dominant cardiolipin species. The SO diet had increased $LA_4CL$, compared to the LD diet ($p=0.0042$). Compared to the SO diet, the LD diet had an increased mean percent for all other cardiolipin species in table 4.4 except for $LA_3AA_1CL$.

**Table 4.6 Fatty Acid Composition of PC**

| Fatty Acid* | LD | SO | p-value |
|---|---|---|---|
| PA (C16:0) | 24.72 ± 0.21 | 21.79 ± 0.14 | <0.0001 |
| SA (C18:0) | 15.10 ± 0.24 | 16.50 ± 0.31 | 0.0019 |
| OA (C18:1) | 8.31 ± 0.17 | 4.76 ± 0.13 | <0.0001 |

| | | | |
|---|---|---|---|
| LA (C18:2) | 11.35 ± 0.35 | 16.46 ± 0.45 | <0.0001 |
| AA (C20:4) | 23.23 ± 0.32 | 26.41 ± 0.34 | <0.0001 |
| DHA (22:6) | 9.12 ± 0.20 | 2.72 ± 0.09 | <0.0001 |

**Table 4.6 Fatty Acid Composition of PC**

Fatty acid composition of PC. N=12 per group. Data presented as mean percent ± SEM. A two-sample t-test was used to determine significance. Significance was determined at P<0.05. * Other FAs not listed include (C14:0, C16:1n7, C18:1n7, C18:3n6, C20:0, C20:1n9, C20:2n6, C20:3n6, C22:0, C22:4n6, C22:5n6, C22:5n3, C24:0, and C24:1n9)

**Table 4.7 Fatty Acid Composition of PE**

| Fatty Acid* | LD | SO | p-value |
|---|---|---|---|
| PA (C16:0) | 17.12 ± 0.40 | 14.59 ± 0.24 | <0.0001 |
| SA (C18:0) | 19.60 ± 0.54 | 21.54 ± 0.48 | 0.0132 |
| OA (C18:1) | 9.90 ± 0.51 | 7.90 ± 0.33 | 0.0030 |
| LA (C18:2) | 5.65 ± 0.48 | 10.39 ± 0.65 | <0.0001 |
| AA (C20:4) | 27.79 ± 0.61 | 28.58 ± 0.67 | 0.3912 |
| DHA (22:6) | 13.33 ± 0.35 | 4.19 ± 0.15 | <0.0001 |

**Table 4.7 Fatty Acid Composition of PE**

Fatty acid composition of PE. N=12 per group. Data presented as mean percent ± SEM. A two-sample t-test was used to determine significance. Significance was determined at

P<0.05. * Other FAs not listed include (C16:1n7, C18:1n7, C18:3n6, C20:0, C20:1n9, C20:2n6, C20:3n6, C22:0, C22:4n6, C22:5n6, C22:5n3, and C24:1n9)

**Table 4.8 Fatty Acid Composition of PS**

| Fatty Acid* | LD | SO | p-value |
|---|---|---|---|
| PA (C16:0) | 9.47 ± 0.24 | 8.61 ± 0.25 | 0.0196 |
| SA (C18:0) | 36.86 ± 0.61 | 36.87 ± 0.65 | 0.9912 |
| OA (C18:1) | 4.13 ± 0.38 | 2.88 ± 0.24 | 0.0101 |
| LA (C18:2) | 7.44 ± 0.90 | 9.98 ± 1.01 | 0.0731 |
| AA (C20:4) | 33.55 ± 0.60 | 34.57 ± 0.61 | 0.2444 |
| DHA (22:6) | 3.54 ± 0.11 | 0.94 ± 0.04 | <0.0001 |

**Table 4.8 Fatty Acid Composition of PS**

Fatty acid composition of PS. N=12 per group. Data presented as mean percent ± SEM. A two-sample t-test was used to determine significance. Significance was determined at P<0.05. * Other FAs not listed include: (C16:1n7, C18:1n7, C18:3n6, C20:0, C20:1n9, C20:2n6, C20:3n6, C22:0, C22:4n6, C22:5n6, C22:5n3, and C24:1n9)

**Table 4.9 Fatty Acid Composition of PG**

| Fatty Acid | LD | SO | p-value |
|---|---|---|---|
| PA (C16:0) | 18.19 ± 0.70 | 17.67 ± 0.52 | 0.5923 |

| | | | |
|---|---|---|---|
| SA (C18:0) | 16.63 ± 0.54 | 16.86 ± 1.16 | 0.8596 |
| OA (C18:1) | 8.92 ± 0.27 | 7.13 ± 1.58 | 0.2765 |
| LA (C18:2) | 34.35 ± 0.59 | 49.40 ± 0.92 | 0.0002 |
| AA (C20:4) | 7.56 ± 0.50 | 8.62 ± 0.47 | 0.6595 |
| DHA (22:6) | 3.67 ± 0.37 | 0.87 ± 0.10 | <0.0001 |

**Table 4.9 Fatty Acid Composition of PG**

Fatty acid composition of PG. N=12 per group. Data presented as mean percent ± SEM. A two-sample t-test was used to determine significance. Significance was determined at P<0.05. * Other FAs not listed include: (C18:1n7, C20:2n6, C20:3n6, C22:4n6, C22:5n6)

**Table 4.10 Fatty Acid Composition of CL**

| Fatty Acid | LD | SO | p-value |
|---|---|---|---|
| PA (C16:0) | 19.84 ± 0.48 | 17.67 ± 0.52 | 0.0056 |
| SA (C18:0) | 18.91 ± 0.65 | 17.97 ± 0.60 | 0.2994 |
| OA (C18:1) | 9.24 ± 0.65 | 4.77 ± 0.50 | <0.0001 |
| LA (C18:2) | 35.03 ± 0.66 | 43.20 ± 0.92 | <0.0001 |
| AA (C20:4) | 7.56 ± 0.50 | 8.62 ± 0.47 | 0.1345 |
| DHA (22:6) | 2.77 ± 0.10 | 0.74 ± 0.04 | <0.0001 |

**Table 4.10 Fatty Acid Composition of CL**

Fatty acid composition of CL. N=12 per group. Data presented as mean percent ± SEM. A two-sample t-test was used to determine significance. Significance was determined at P<0.05. * Other FAs not listed include: (C18:1n7, C20:2n6, C20:3n6, C22:4n6, C22:5n6)

## 4.11 Hepatic Phospholipid Fatty Acid Composition

The main fatty acids of the phospholipids are listed as mean percent of the total fatty acids identified (Tables 4.6 – 4.10). PA was largest percentage of PC for the LD diet group, and AA was the largest percentage for the SO diet. PA was significantly different between diets in all phospholipid fractions reported, except for PG (p-value:0.5923 SA was the largest percent in PS, for both diet groups. SA was increased in the SO diet for PC and PE phospholipid fractions(p-value:0.0019,0.0132). LA was the highest percent in both diet groups for PG and CL. LA was increased in all phospholipid fractions for the SO diet except for PS (p-value:0.0731). OA was significantly increased in the LD diet for all phospholipid fractions except PG (p-value:0.2765). AA was increased in the SO diet compared to the LD diet in the PC phospholipid fraction (p-value: <0.0001). DHA was significantly increased in all phospholipid fractions in the LD diet compared to the SO diet (p-value: <0.0001).

# Chapter 5: Discussion

Non-alcoholic fatty liver disease is defined as hepatic fat accumulation that occurs without the consumption of alcohol (1). Risk factors for NAFLD include obesity, hyperglycemia, T2DM and insulin resistance (1,26, 27). Past research indicates that altered fatty acid composition of hepatic lipids, and plasma phospholipids occur in both animal models and human subjects with NAFLD (6-8,35, 48, 132). Hepatic mitochondrial dysfunction has been described in NAFLD/NASH as damaged mitochondrial DNA, decreased hepatic mitochondria respiratory complex activity, and increased generation of reactive oxygen species (27, 35-38). Here we assessed the effect between dietary fat on hepatic phospholipid compositions in a mouse model of diet induced obesity. The mice were fed a high diet of either a LA-rich safflower oil or SF-rich lard diet with the main goal to determine any changes in fatty acid compositions of the hepatic phospholipids. Both diets exhibited distinct hepatic phospholipid fatty acid profiles (Table 4.6-4.10). We also found that SO diet had increased $LA_4CL$ compared to the LD diet (Table 4.5). The SO diet had decreased mean blood glucose between the 90 minute and 120-minute time point (Figure 4.3). The Δglucose AUC was trending towards significance compared to the LD diet (Figure 4.4). The SO diet was found to have a higher hind limb force than the LD diet (Figure 4.8).

**5.1 No significant differences in mean body weight and food intake after 130 days**

Past clinical studies found that increased PUFA intake may result increased lean mass, and lower visceral, liver fat compared to SFA (11,63,64,65, 133,134). Additionally, both

animal and clinical studies have shown that increased LA intake was associated with decrease in adipose accumulation (11,61,65,133,134). In the current study, body weights were significantly lower in the SO diet compared to the LD diet from day 28 to day 75, as was total food intake from day 31 to day 69 and adiposity at day 65. This significant difference coincided with the limiting food exchange to twice per week from every two days due to the Covid-19 pandemic. This factor contributes to the decrease in food intake in the SO diet which led to the lower body weight and smaller increase in adiposity seen in the SO diet. We have reason to believe this is the cause of the significance between the diet groups, because at day 100 we calculated no significant different in change in adiposity when total food and body weight were not significant different at this time point. We theorized that since the SO diet was predominantly LA, it may have oxidized faster than the LD diet, which was predominantly SF and MUFA, which led to decrease in food intake in the SO diet group.

**5.2 The SO diet had increased hind limb force compared to the LD diet group**

LA consumption has been linked to increase in lean mass in several clinical studies (63,66,67, 133). Decreases in lean mass, which is notably mostly comprised of muscle mass has been linked to increase risk of chronic disease, impaired muscle function, and loss of muscle strength (135). Grip strength is a way to assess skeletal muscle function in mice. In the present study, although fore limb force was not significantly different between the LD diet group and SO diet group. However, hind limb force was significantly higher in the SO diet group compared to the LD group (Figure 4.8). This was without a significant increase in lean mass between diet groups at day 100 (Figure

4.10). LCFAs and PUFAs were discovered to be natural ligand for PPAR-β/δ in CV-1 cells (136,137,138). PPAR- β/δ activation has been discovered to protect PGC-1α degradation in skeletal muscle (139). PGC-1α plays a central role in cellular metabolism and stimulates mitochondrial biogenesis (140). Knocking down of PPAR-β resulted in large decreases of PGC-1α protein in skeletal muscle (139). High dietary fat intake has been shown to decrease expression of genes in skeletal muscle relating to oxidative phosphorylation and PGC1-α, suggesting that high fat diets can disrupt mitochondrial function and biogenesis (141). Although not measured in the current study, the SO diet group may be benefiting from the increase intake of LA, by increased activation of PPARs and PGC-1α, which could restore mitochondrial biogenesis and function. Future studies should determine if the SO diet can increase PGC-1α content or expression of PPAR-β and improve mitochondria function. In rat model studying hypertensive heart failure, found that the feeding of a high LA safflower oil restored $LA_4CL$ concentrations in hearts (142). On the other hand, high fat feeding can decrease mitochondrial respiration and ATP synthesis in skeletal muscle of mice (143). $LA_4CL$ seems vital to ATP synthesis in all tissues that exhibit oxidative capabilities and is associated with improved mitochondrial ATP synthesis by increased ATP synthase activity, and super complex formation (68,74,96,97). Replacement of LA in CL species by DHA also lead to impaired respiratory complex activity of I, IV,V I+III in the heart (144). While the mice on the SO diet did not have more muscle mass, they may have more functional mitochondria and muscle tissue compared to the LD diet group. PPAR-β/δ and PGC-1α

in the skeletal muscle could be analyzed through PCR to determine any changes in expression.

### 5.4 SO diet exhibited lower blood glucose at 90 minute and 120-minute time points compared to the LD diet

Several clinical studies have shown improvements with increased consumption of safflower oil, and increased serum linoleic acid decreased HbA1c, blood glucose and increased lean mass (62,63-65, 133). In a multiple treatment meta regression analysis found that replacement of SFAs with PUFAs lowered HbA1c, CRP and HOMA IR scores in humans (62). Although, Fasting blood glucose was not significantly between the two diet groups on both day 85 and 91 of our ITT and GTT. Our ITT data suggests that the mice consuming the LA rich diet have improved glucose clearance than the LD diet group. In a study with obese rats, found that addition of 10% increase LA, was able to attenuate whole body glucose intolerance and improve insulin tolerance in skeletal muscle (145). This was found independent from changes in oxidative stress, oxidative phosphorylation proteins and antioxidant enzyme gene expression (145). This implies that increased LA consumption is improving insulin sensitivity in a different way, not necessarily by improved mitochondrial function.

### 5.5 No Differences in TG and Total Lipid Content

Two clinical studies have reported when compared against SFAs, increased LA intake has been linked reduced liver fat, despite weight gain in the overfeeding study, and without significant weight loss in the other (66,67). In our mice, we had no significant differences in TG, and total lipid content between the two diets. This may suggest that

dietary fat, specifically increased LA may not be able to stop accumulation of fat in the liver from long term intake of high fat diet. A study in rats being feed a high fat diet with a ratio of n-6: n-3 of 200 2 or 5, by mixing various source oils like peanut, palmolein, and linseed oil found that the ratios of 2 or 5 both had significant decrease in liver weight compared to n-6/n-3 ratio of 200 (146). These results suggest that ALA was able to attenuate insulin resistance, lower inflammation, and decreased triglyceride content compared n-6:n-3 ratio of 200 (146). A study in 2017 found that mice fed on a high fat, high oleic safflower oil diet supplemented with fish oil, but not a high fat, high butter diet supplemented with fish oil had exacerbated sucrose induced fatty liver, and increased expression of PPARγ1 &PPARγ2 and CD36 mRNA in the high safflower oil fed mice (146). In the high oleic safflower oil diet, they also found slight increases of some PPAR-α target genes such as acetyl-CoA carboxylase, CPT-1 and medium chain acyl-CoA dehydrogenase (MCAD), but only the increase in MCAD was significant (146). These two animal studies imply that dietary fat plays a role in fatty liver development and that the replacement or supplementation of PUFAs may be beneficial in specific NAFLD induced models.

**5.6 SO diet increased $LA_4CL$ species and total LA content in CL**

Cardiolipin has several key functions that are vital to proper mitochondrial function like anchoring of mitochondrial proteins, facilitating proton leakage, mitochondrial fusion/fission, and overall mitochondrial architecture (68-70, 72). The dominant cardiolipin species in the hepatic mitochondria are listed in Table 4.4, and their percent of total species is in Table 4.5. The LD diet had increased $LA_3OA_1$ CL and $LA_2OA_2$ CL

species. Which was expected since the LD diet contained more oleic acid than the SO diet. On the other hand, SO diet had a higher percent of $LA_4CL$compared to the LD diet. The SO diet had increase in the $LA_3AA_1$ CL species compared to the LD diet in mice. Lipidomic analysis of hepatic CL species in BXD mice found that increased $LA_4CL$ and MLCL, were negatively correlated with obesity and traits of NAFLD such as (increased liver TG, increased body weight, fasting glucose) (147). Given the increase of $LA_4Cl$ in the SO diet we were hoping to see a decrease in hepatic lipid content, maybe indicating that the SO livers had healthy mitochondria while consuming a high fat diet. Since depletion or the replacement of LA in cardiolipin species by other fatty acids has been linked to lower mitochondrial function in the heart, we theorized that maybe this process was involved in the development of mitochondrial dysfunction in NAFLD. In study examining cardiolipin speciation in mice fed a control diet and high fat diet found that high fat diet did not display decreased remodeling enzymes of cardiolipin but found increased hepatic $LA_4CL$ (148). The increased expression of TAZ the livers was also increased which the authors suggested that this contributed to the increase $LA_4CL$ (148). Since the mice in our study were also fed a high fat diet for a similar amount of time, future analysis should examine the gene expression of TAZ to determine if this contributed to the difference in hepatic $LA_4CL$. Another study in rat liver mitochondria found an acyl transferase that does not require activation of FAs like through the Land's Cycle, was able to transfer LA chains from PC and PE to CL (149). It is believed that TAZ, is a part of this single acyl transfer as the trans acylase activity was decreased with the deletion of TAZ (149). The transfer did not require activation by CoA which is

required by remodeling by genes ALCAT1 and MLCLAT1 (91, 98,149). The SO diet group also had increased percent of LA compared to LD, into PC and PE phospholipids (Table 4.6 & 4.7). This suggest that if the TAZ activity and availability of LA is increased, then this may result in the increase of the $LA_4CL$ in the SO diet. It should be stated that TAZ enzymatic reactions are reversible and can react with of phospholipid classes with variety of fatty acids (150). A newly proposed theory suggests that TAZ is responsible for creating membranes with negative curvature with phospholipids such as CL to support protein packing (11,68,150). This may imply that inclusion of $LA_4CL$ may help support super complex formation, along with creating an environment forcing TAZ to interact only with LA rich CL rich membranes. More analysis is still required to confirm this theory since both diets are high fat diets and may have similar TAZ expression, but the LD diet will lack the substrate availability of LA compared to the SO diet and may lack distinctive membrane properties that may inhibiting TAZ activity.

ALCAT1 recognizes MLCL and dilysocardiolipin (DLCL) as substrates, and its remodeling activity has been linked to increased oxidative stress, mitochondrial dysfunction in cardiac tissue (91). Its overexpression led to massive increasing of DHA incorporation into CL in C2C12 myoblast cell lines, at the expense of LA (91). Increase DHA in mitochondrial membranes have been linked to increased membrane lipid oxidation and altered cellular membrane composition that promotes apoptosis (145). LD diet had increased DHA compared to SO diet (Table 4.5). Besides increased incorporation of DHA, ALCAT1 also can use Oleoyl-CoA and Linoleoyl-CoA as acyl donors (98). In the upregulation of ALCAT1 could be the reason we seen an increase in

$LA_3OA_1$ CL and $LA_2OA_2$ CL species in the LD mice. Besides the difference in OA content in the diets, OA can be synthesized in animals from the elongation of palmitic acid, by ELOVL6 to stearic acid and then synthesized by Δ9-deasaturase (149). Since our SO diet was predominantly LA, if ALCAT1 had increased expression it may only increase the $LA_4CL$ species, while in LD diet it could potentially increase $LA_4CL$, as well as $LA_3OA_1$ CL and $LA_2OA_2$ CL species. While these results from this study was C2C12 myoblast cell lines, in mice ALCAT1 protein expression was dramatically increased in severe NAFLD in wild type mice after 18 weeks of high fat feeding (90). When ALCAT1 was knocked out in mice, mice had increased insulin sensitivity, glucose tolerance, decreased hepatic triglycerides and liver weight (90). ALCAT1 knock out mice also had significantly restored $LA_4CL$ species in the heart (91). Future analysis for this study should analyze hepatic ALCAT1 to determine if there are any differences in mRNA content between SO and LD group.

**5.7 Both diets had distinct hepatic phospholipid fatty acid profiles**

Fatty acid compositions of phospholipids are listed in tables (4.6 – 4.10). In these tables we found the main fatty acids were palmitic acid (PA), stearic acid (SA), oleic acid (OA), linoleic acid (LA), arachidonic acid (AA) and docosahexaenoic acid (DHA). Overall, we found that certain fatty acids were of similar between groups despite the difference in dietary fat source. As AA was a dominant species in PC, this makes sense as the cleaving of AA from PC can be used to synthesize the eicosanoids, as potent anti/inflammatory signal molecules (50,152). In addition, each fatty acid listed in table 4.6 was significantly different between the two diets for PC. This is not a surprise since PC, accounts for about

~50% of total phospholipids in the bilayer of many organelles and is synthesized in large quantities in the liver for lipoproteins and bile (104-105).

In the SO diet had increased SA incorporation into PC and PE, despite the difference in SA content between the two diets. Increased dietary stearic acid was shown to help reduce mitochondrial fragmentation by covalent binding (stearoylation) to transferrin receptor (TfR1) to inhibit JNK signaling to block mitochondrial fusion (149). In this same study, they reported that lower circulating SA levels had reduced mitochondrial respiration, theorized to reduced beta oxidation, and the increased presence of acyl carnitines in the blood (149). Acyl carnitines profile test can be used to determine inherited disorders in fat catabolism (154). While used for inherited diseases, metabolic diseases that affect lipid metabolism, may cause increased acyl carnitines concentrations in the blood. Lipidomic analysis of purified mitochondria from drosophila model, found that mitochondria do not contain much SA in their membranes, suggesting it does not play a structural role (153). A pilot study with ten human subjects found that upon digestion of dietary SA causes mitochondrial fusion within a couple hours (155). However, the role of SA in phospholipids is not discussed in depth in these papers, but it is quite puzzling that despite lower dietary intake of SA from the SO diet there was increased incorporation of SA into phospholipids fractions of PC and PE compared to LD mice. SA was not significantly different between the diets for PS, PG and CL.

OA content of the phospholipid fractions was increased in PC, PE, PS and CL in the LD diet compared to the SO diet. In all phospholipid fractions, OA was <10% of total fatty acids measured. However, since the fatty acids are reported as percent of total, 10%

of PC is probably a higher concentration when compared to 10% of smaller phospholipids. As a non-essential fatty acid, OA may play a big role as a metabolite and not as a structural fatty acid for phospholipids. In a study conducted, in 2017 found that a diet rich in OA fed to wild type mice found activation of Liver X receptors (LXR) reduced cholesterolemia, while in knocked out Liver X receptor (LXR) mice induced signs of inflammation liver damage (156). LXR is directly involved in hepatic lipogenesis and suggesting that OA activating LXR can protect the liver from inflammation while inducing lipogenesis (156).

LD diet had increased DHA incorporation into all phospholipid fractions compared to the SO diet. Dietary DHA may be preferentially incorporated into PE, both labeled 13C-DHA, and unlabeled DHA ingestion was found predominantly in PE (157). This study concluded that a possible reason that DHA is preferentially incorporated PE which can be converted to PC to be delivered to the brain in times of low n-3 consumption (157).

# Epilogue

## Conclusions

This study assessed if an LA-rich safflower oil diet could alleviate hepatic lipid accumulation that is essential in the development of NAFLD. The SO diet had lower blood glucose measurements during the ITT, along with Δglucose AUC trending towards significance. The SO diet mice had increased hind limb force compared to the LD diet. The SO diet had increased $LA_4CL$ species of CL. The SO diet mice had increased LA integration into all phospholipids except for PS compared to the LD diet. These results suggest that while dietary fat source does affect hepatic phospholipid fatty acid composition; high fat feeding of LA-rich safflower oil does not alleviate hepatic lipid accumulation compared to LD diet.

## Limitations and Future Directions

There are several limitations to our study. After restrictions were set in place, we were only allowed to continue with experiments after about two months of limited access to our mice. For safety reasons, we limited food exchanges and we have reason to believe that this influenced body composition measurements on day 65, since the SO diet mice had lower food intake and lower body weights at this point. The mice may have increased fasting, due to change in taste we theorized from the oxidation of the food in the SO diet because it is so high in LA which could have affected our other experiments on later days. We do not have baseline grip strength measurements to compare force between the two diets, which could help determine if the changes of force we measured are replicable and if there was a difference between baseline and measurements on day 113. Since we

used safflower oil and lard not specifically diets enriched with just LA or PA, there could be other phytochemicals in safflower oil that have beneficial properties. For example, safflower oil can be rich source of Vitamin E, as the oils range from ~46 – 70 mg/100g (158). At the end of this study, we are not certain that our mice had developed fatty livers, and its unknown whether consuming a high fat diet rich in LA diet would impose any benefits to healthy livers.

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## Supplemental Figures

**Figure 13 Dominant Fatty Acids in PC**

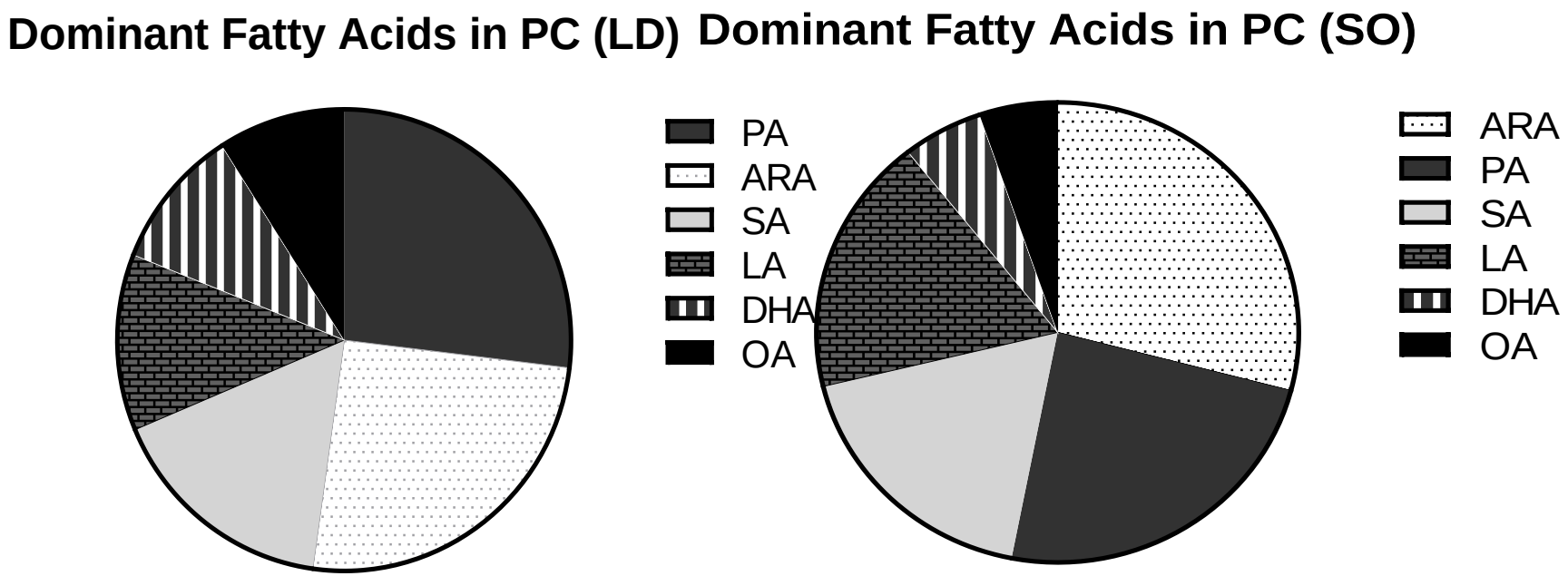


**Figure 14 Dominant Fatty Acids in PE**

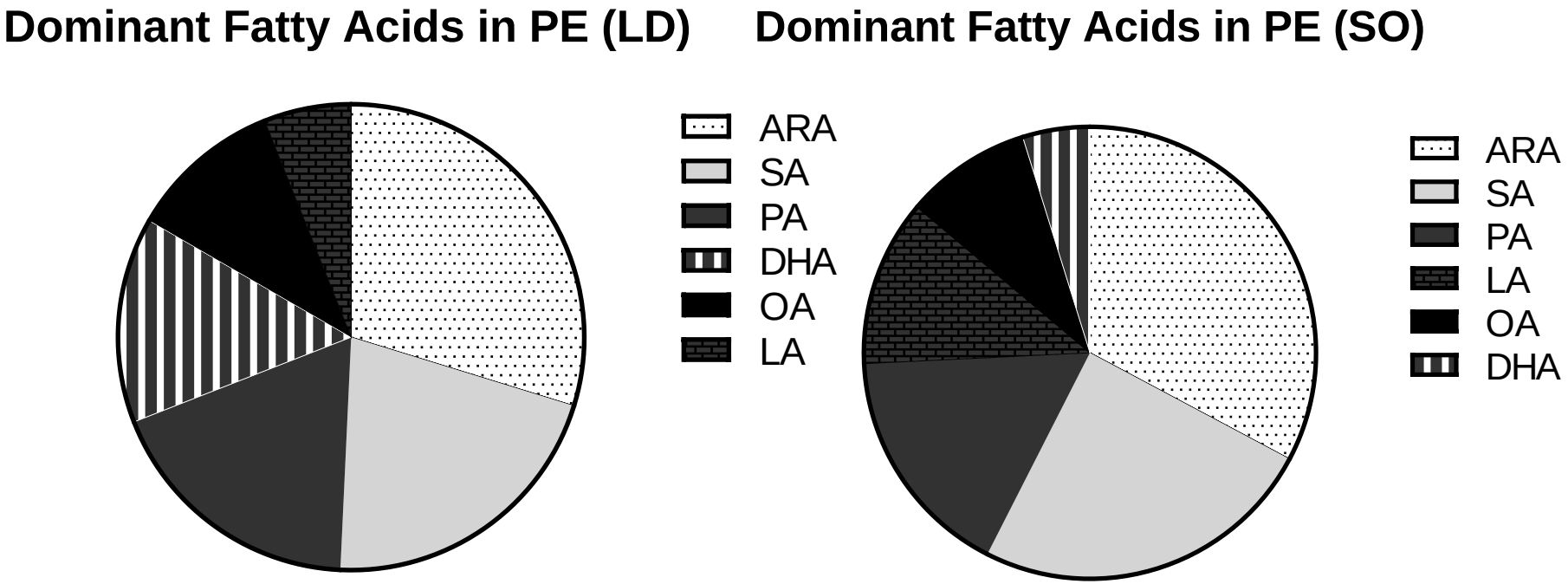
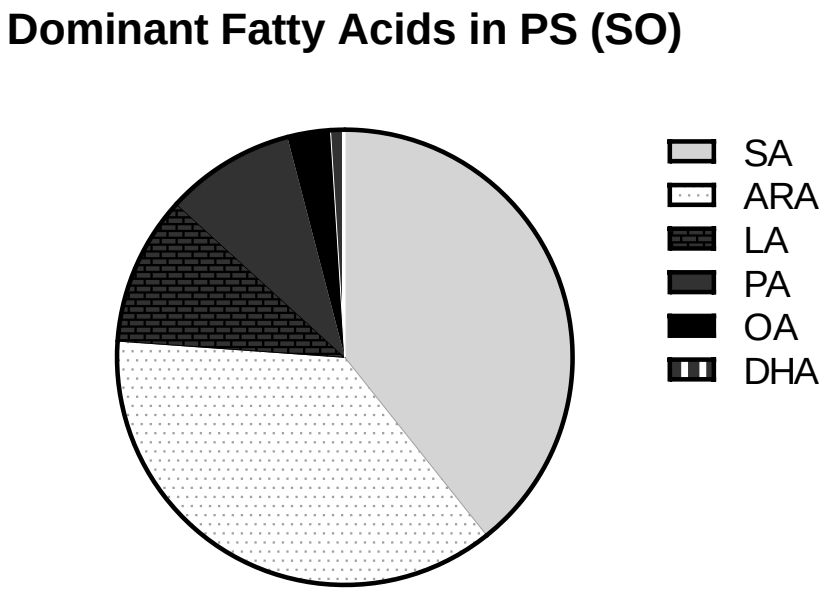


**Figure 15 Dominant Fatty Acids in PS**



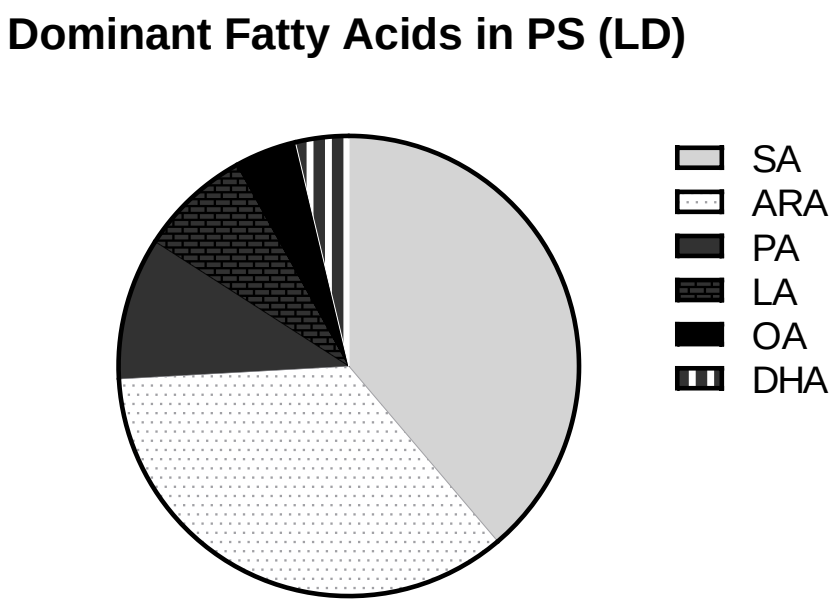



**Figure 16 Dominant Fatty Acids in PG**

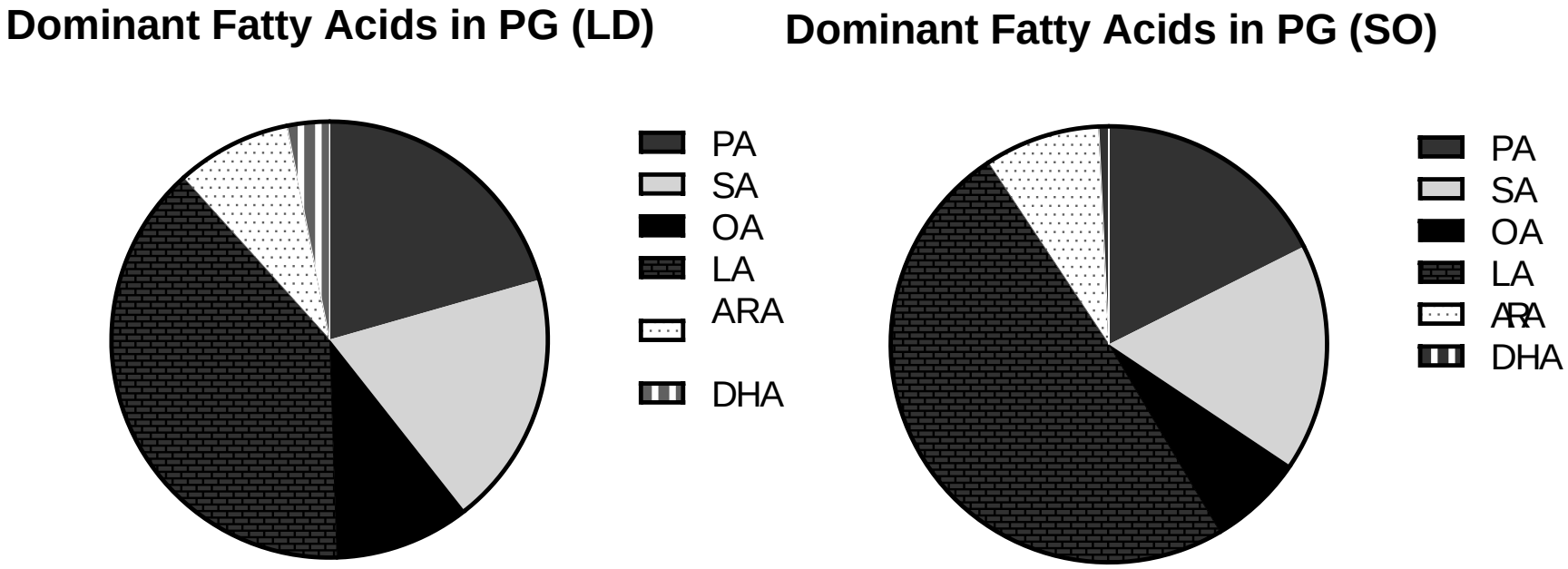


**Figure 17 Dominant Fatty Acids in CL**

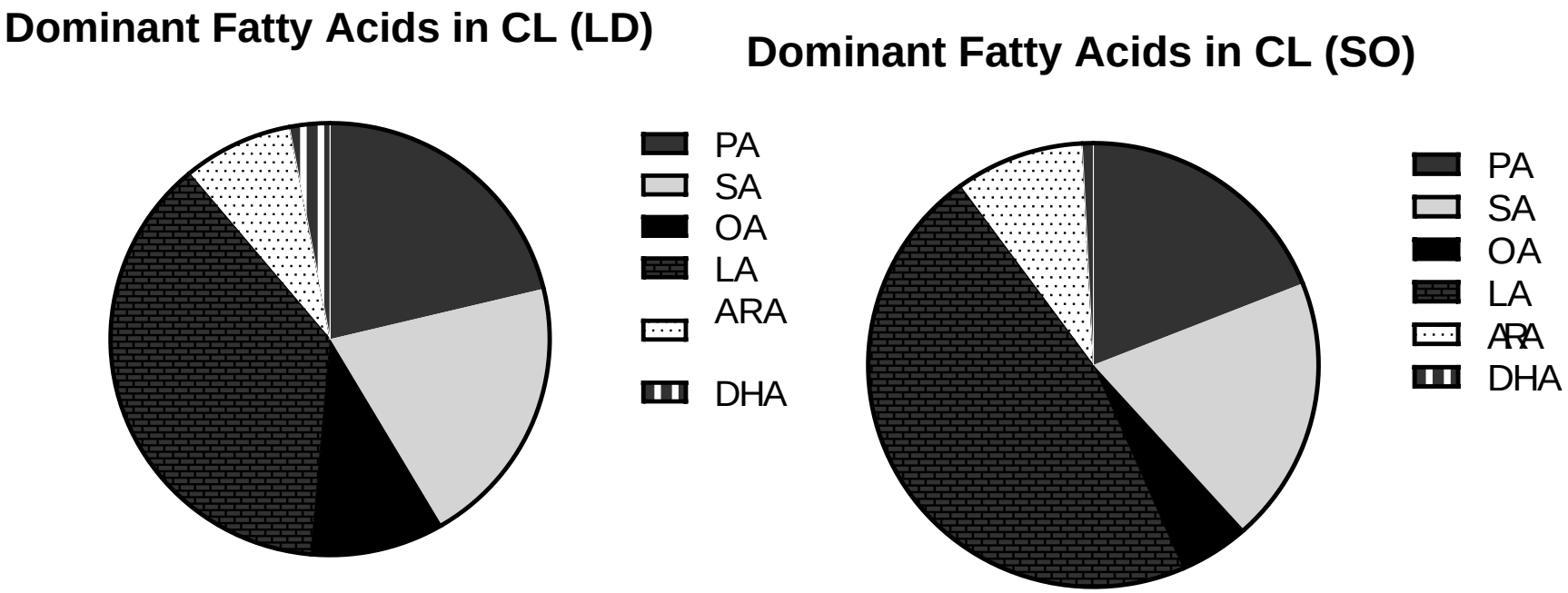


**Figure 18 Food Efficiency Ratio**