The Effects of Plant versus Marine Sources of Dietary Omega-3 Fatty Acids on Hepatic Steatosis and Adipose Function in *fa/fa* Zucker rats

by

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A Thesis submitted to the Faculty of Graduate Studies of

The University of Manitoba

in partial fulfillment of the requirements of the degree of

MASTER OF SCIENCE

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ABSTRACT

Non-alcoholic fatty liver disease (NAFLD) is a common consequence of metabolic syndrome (MetS) with the least severe form of NAFLD being hepatic steatosis, which is the accumulation of intrahepatic fat. Omega-3 polyunsaturated fatty acids (n3 PUFAs) are fatty acids in our diets commonly found in marine animals (eicosapentaenoic acid [EPA] and docosahexaenoic acid [DHA]) and certain plants (α-linoleic acid [ALA]). Although past studies have examined the consumption of marine sources or plant sources on hepatic steatosis and MetS parameters, individual n3 PUFA have yet to be compared to each other. Thus *fa/fa* Zucker rats were provided n3 PUFA diets containing ALA, EPA or DHA for 8 weeks relative to a linoleic acid (LA)-rich n6 PUFA diet provided to *fa/fa* and lean Zucker rats. Comparisons were to baseline *fa/fa* Zucker rats. It was shown that DHA prevented the progression of hepatic steatosis and was associated with improvements in insulin resistance.

ACKNOWLEDGEMENTS

First and foremost I must thank my two advisors Dr. Carla Taylor and Dr. Peter Zahradka for all their time, guidance and encouragement through this experience. I am thankful that I have had the opporitunity to be part of their labs and I could not have asked for better advisors to work with. Thanks to Dr. Harold Aukema and Dr. Grant Hatch for being on my committee; your comments and suggestions were greatly appreciated. I would also like to acknowledge funding for the project from the Agri-Food Research and Development Initiative and scholarship support the NSERC-CREATE Food Advancement through Science and Training (FAST) program and the University of Manitoba Graduate Scholarship program.

Thanks to the R.O Burell Laboratories along with Amy, Danielle, Jaime and Megan for all their help and assistance with animal care. I couldn't have done this without your help. Thanks to Dennis Labossiere for GC work. Thanks to Dr. Jeffery Wigle for all your help with imaging and your patience with me while using the Zeiss Axioskop 2 plus microscope. Also a huge thanks to the Zahradka/Taylor lab team, you have made my 2 years here an enjoyable experience. You guys are the best.

Finally, I would like to issue a huge thanks to all my family and friends for all your support and encouragement throughout the course of my Master's.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF APPENDICES

LIST OF ABBREVATIONS

ACC	Acetyl-CoA carboxylase
ACO	Acetyl-CoA oxidase
ACE	Angiotension-converting enzyme
ANOVA	Analysis of variance
ALA	α-linoleic acid
ALT	Alanine transaminase
ALP	Alkaline phosphatase
APS	Ammonium persulfate
AST	Aspartate transaminase
ATP	Adenosine triphosphate
AUC	Area under the curve
BHT	Butylated hydroxytoluene
BMI	Body mass index
C	Corn starch
Ca	Canola oil
CA	Corn starch+chia
Ca/F	Canola/flax
CE	Corn starch+eicosapentaenoic acid
CD	Corn starch+docosahexanoic acid
CRP	C-reactive protein
CTL	Control
Cu	Copper
ddH_2O	Double distilled water
DHA	Docosahexaenoic acid

DIO	Diet induced obesity
DM2	Diabetes mellitus type 2
DNM	Did not measure
DOCA	Deoxycorticosterone acetate
DPA	Docosapentaenoic acid
eEF2	Eukaryotic translation elongation factor 2
EPA	Eicosapentaenoic acid
FA	Fatty acid
faALA	*fa/fa* Zucker rats fed diet containing n3 from ALA
faBASE	Baseline *fa/fa* Zucker
faDHA	*fa/fa* Zucker rats fed diet containing n3 from DHA
faEPA	*fa/fa* Zucker rats fed diet containing n3 from EPA
faLA	*fa/fa* Zucker rats fed diet containing n6 from LA
FFA	Free fatty acid
FGF-21	Fibroblast growth factor-21
FL	Lean fed flax oil
FO	Obese fed flax oil
FXO	Flaxseed oil
GC	Gas chromatography
GGT	Gamma-glutamyl-transpeptidase
GOD-POD	Glucose oxidase-glucose peroxidase
H	High fat
HA	High fat+chia
HCl	Hydrochloric acid

HE	High fat+eicosapentaenoic acid
HD	High fat+docosahexanoic acid
HF	High fat diet
HFO	High fat fish oil diet
HIF	Hypoxia inducible factor
HIG2	Hypoxia-inducible protein 2
HDL-C	High-density lipoprotein cholesterol
HFD	High fat diet
HOC	High oleic canola oil
HOMA-IR	Homeostasis model assessment of insulin resistance
HPLC	High performance liquid chromatography
HRP	Horseradish peroxidase
IL-1B	Interleukin-1B
IL6	Interleukin 6
Kcal	Kilocalorie
Kg	Kilogram
L	Lard
LA	Linolenic acid
LL	Lean fed lard
LLOD	Lower limit of detection
LDL-C	Low density lipoprotein cholesterol
LO	Obese fed lard
LPL	Lipoprotein lipase
NaCl	Sodium chloride
n3	Omega3

n6	Omega6
NAFLD	Non-alcoholic fatty liver disease
NASH	Non-alcoholic steatohepatitis
ND	No difference
nm	Nanometer
MCP-1	Monocyte chemoattractant protein
MAP	Mean arteriole pressure
MetS	Metabolic syndrome
mg	Milligrams
mmHg	Millimeters of mercury
MSD	Meso-Scale Discovery
MO	Menhaden oil
mRNA	Messenger ribonucleic acid
MUFA	Monounsaturated fatty acid
OCT	Optimal cutting temperature
OGTT	Oral glucose tolerance test
OP-CD	Obese prone-Charles River Sprague Dawley
pACC	Phosphorylated acetyl-CoA carboxylase
pACO	Phosphorylated acetyl-CoA oxidase
PBS	Phosphate buffered saline
PBS-T	Phosphate-buffered saline with Tween-20
PL	Phospholipid
PPARα	Peroxisome proliferator-activated receptor alpha
pPPARα	Phosphorylated peroxisome proliferator-activated receptor alpha

PTP1B	Protein tyrosine phosphatase 1B
PUFA	Polyunsaturated fatty acid
PVDF	Polyvinylidene difluoride
QMR	Quantitative Magnetic Resonance
SAS	Statistical Analysis Software
SEM	Standard error of the mean
SHR	Spontaneously hypertensive
SC	Standard chow
SDA	Steradonic acid
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate -polyacrylamide gel electrophoresis
SF	Safflower
SFA	Saturated fatty acid
TAG	Triglyceride
TBST	Tris-buffered saline with Tween-20
TEMED	N,N,N’,N’-tetramethylethylenediamine
TLC	Thin layer chromotography
TNF-α	Tumor necrosis factor-alpha
TUP	Theoretical upper phase of partition
SDA	Stearidonic acid
SREBP	Sterol regulatory element binding protein
VLDL	Very low density lipoprotein
VPR	Volume pressure recording
WM	Weight matched

LITERATURE REVIEW

Introduction

Non-alcoholic fatty liver disease (NAFLD) is a common consequence of metabolic syndrome (MetS) with the least severe form of NAFLD being hepatic steatosis, which is the accumulation of intrahepatic fat. Over time, steatosis can progress to more severe forms, but even if it does not there are various consequences associated with this condition, such as insulin resistance, hypertriglyceridemia, and lower levels of high-density lipoprotein cholesterol (HDL-C) (reviewed by Garg and Misra, 2002). Overweight is defined as having a body mass index (BMI) from 25.0-29.9 and obesity is defined as having a BMI of $\geq$ 30. Being overweight or obese is associated with many chronic diseases including, NAFLD, MetS, diabetes mellitus type 2 (DM2) and dyslipidemina (Chalasani et al., 2012).

Studies have shown that patients with hepatic steatosis present a lower n3/n6 polyunsaturated fatty acid (PUFA) ratio in liver tissue biopsies, namely in phospholipid (PL) subfractions and in red blood cells (reviewed by Shapiro et al., 2011). There is also evidence that supplementary consumption of n3 PUFAs in animal models and in human interventions results in improvement of NAFLD symptoms and consequences (reviewed by Di Minno et al., 2012).

n3 PUFAs are fats that are essential in our diet because the human body cannot synthesize them. Despite the fact that humans cannot synthesize n3 PUFAs *de novo*, we are able to convert shorter chained n3 PUFAs, such as α-linoleic acid (ALA, C18:3 n3), to longer chained n3 PUFAs, such as eicosapentaenoic acid (EPA, C20:5n3) and docosahexaenoic acid (DHA, C22:6n3), via elongation and desaturation (reviewed by Deckelbaum & Torrejon, 2012). ALA is found in plant sources such as canola and

flaxseed oils, while EPA and DHA are found in marine sources and algae. Docosapenatenoic acid (DPA, C22:5n3) is another marine source of n3 PUFAs found primarily in seal oil, but its biological properties are not as thoroughly studied due to limited availability of the purified form (Kanayasu-Toyoda, 1996; Kaur et al., 2010)

Even though there are studies that investigated the effects of n3 PUFAs from marine sources on obesity and hepatic steatosis (reviewed later in this proposal), investigations of dietary plant-based n3 PUFAs and their effects are more limited. The purpose of this thesis is to directly compare plant versus marine n3 PUFA sources on hepatic steatosis, MetS and adipose function in a rodent model of obesity.

Hepatic Steatosis

NAFLD is clinically defined as having a liver triglyceride (TAG) content exceeding 5% of the liver weight (reviewed by Garg and Misra, 2002). This diagnosis is made in the absence of excessive alcohol intake, or evidence of viral, autoimmune or drug-induced liver disease (reviewed by Yeh and Brunt, 2007). NAFLD includes a spectrum of liver diseases, formally known as hepatic steatosis, where accumulation of intrahepatic fat occurs. NAFLD can progress from simple hepatic steatosis to more a severe condition involving necrotic inflammation and fibrosis known as non-alcoholic steatohepatatis (NASH)(Aly and Kleiner, 2011).

With the more recent shifts in eating patterns and lifestyle, there has been an alarming rise of children and adults falling into unhealthy weight classes. According to a recent survey, 1 in 3 Canadian children and 2 in 3 adults were classified as overweight or obese (Statistics Canada, 2011). Due to the alarming rise in overweight and obese

children, it is estimated between 3-8% of North American children currently have NAFLD (reviewed by Giorgio et al., 2013). It is also reported that 44% of obese Italian children and adolescents and 74% of obese Chinese children and adolescents (aged 2-19) were affected with a liver disorder related to NAFLD (reviewed by Manco et al., 2008). Being overweight or obese increases the risk of developing many chronic diseases, including MetS. MetS is a term used to describe a group of conditions that put people at higher risk of developing chronic diseases such as DM2, coronary heart disease and other heart-related problems (NIH Heart Lung and Blood Institute, 2011). NAFLD is associated with obesity and MetS.

Diagnosis

Hepatic steatosis is often asymptomatic, except for nonspecific clinical symptoms or signs such as easy fatigability and right upper quadrant abdominal discomfort (Park et al, 2006). There are also no clinical features that are definitively indicative of hepatic steatosis and other forms of NAFLD in obese children (reviewed by Yang 2013). The gold standard for detection is through a liver biopsy, which is an invasive procedure that is time consuming, costly and potentially uncomfortable for patients, especially paediatric patients (Festi et al., 2013). The most common form of non-invasive detection of NAFLD is the use of ultrasonography, although this method will not show the severity or the liver histology (Feldstein et al., 2010).

Laboratory tests that are routinely used in the evaluation of patients with suspected NAFLD include a serum panel testing for the liver enzymes: alanine transaminase (ALT), aspartate transaminase (AST), alkaline phosphatase (ALP) and

gamma-glutamyl-transpeptidase (GGT). There are also laboratory tests done for albumin, prothrombin coagulation time, and complete blood count. However, the issue for diagnosis is that the detection of elevated levels of liver enzymes, abnormal prothrombin coagulation time and abnormal complete blood count results do not indicate the severity of the condition, and rather only show that there is liver dysfunction (Mofrad et al., 2003).

Other proposed ways of detecting NAFLD, especially in children, are biomarkers for inflammation such as C-reactive protein (CRP) and cytokines (Feldstein et al, 2010). Increased circulating levels of tumor necrosis factor (TNF)-α and interleukin (IL)-6, which are pro-inflammatory cytokines, have been suggested as possible biomarkers for both adults and children with NASH. Increased circulating levels of leptin and decreased circulating levels of adiponectin, which are adipocytokines, are also reported to be related to NAFLD development in adults and children.

Prevention, early detection and management, lifestyle and diet interventions should be effective for minimizing the progression, and even reducing the impact, of hepatic steatosis. If left unmanaged, non-alcoholic hepatic steatosis can progress to liver disease with fibrosis, more formally known as NASH. MetS components such as obesity and insulin resistance are strongly associated with the increase in the prevalence of NAFLD along with worsening the prognosis (Bugainesi al., 2002). Patients with NAFLD who also have components of MetS are also at higher risk for developing a progressive type of NASH (Chalasani et al., 2012).

What is of special interest in comparing NAFLD in children and adults is that the histological appearance of the disease differs significantly, likely representing a physiological response to environmental stressors in children and long-term adaptation in

adults (reviewed by Manco et al., 2008). Through agglomerative hierarchical cluster analysis, Schwimmer et al. (2005) found that based on histological appearance, two forms of NASH are identifiable in children and adults. Type 1 NASH was consistent with NASH as often described in adults and was characterised by steatosis, ballooning degeneration, and/or perisinusoidal fibrosis in the absence of portal change. Type 2 NASH is a peculiar histological pattern in children characterized by steatosis, portal inflammation and/or portal fibrosis in the absence of ballooning degeneration and perisinusoidal fibrosis. They also found that children with type 2 NASH were significantly younger and had a greater severity of obesity than children with type 1 NASH.

Takahashi and Fukosato (2010) compared the histological appearance of liver biopsies from 34 pediatric and 23 adult NAFLD cases in Japan and found that steatosis tended to be more severe in children compared to adults. They found that perisinusoidal fibrosis was significantly less frequent in children than in adults, and intralobular inflammation and ballooning degeneration tended to be milder in children as compared to adults. In summary, children with NAFLD maintain a non-histological detrimental status of NAFLD, allowing for the possibility to revert the progression through lifestyle management (Widhalm and Ghods, 2010).

Early management

The most commonly suggested way to prevent, and even reverse the progression of MetS and hepatic steatosis is through early management, which includes increasing daily physical activity and eating a healthier diet. A study done by Wang et al. (2008)

showed that a short term intervention of a daily exercise routine and a controlled diet had a positive effect on NAFLD, and suggested that it should be the first step in management of children with NAFLD. The experimental group of children went to a supervised summer camp where they were given a calorie-restricted diet in an attempt to reduce daily caloric intake by 250 kcal/day. The diet was rich in lean meats and fish, and low in fat. The children had a daily morning and afternoon aerobic routine that took up a total of 3 hours per day, and a large portion of the day was involvement in recreational sport activities such as swimming and basketball. After 1 month of the intervention the participants had a decrease in BMI, AST and total cholesterol, and 10 of 19 participants had normal liver function. A similar study was done on an adult population with NAFLD and it showed the positive effects of increased physical activity, nutrition and behaviour therapy on parameters such as body weight, BMI and waist circumference (visceral adiposity). The results also showed a decrease in fasting serum ALT, AST, cholesterol, TAG, glucose and insulin (Sun et al. in 2012). The intervention group in the study was given a diet low in fat and simple sugars with an ideal caloric amount of 25-30 kcal/kg. They were also encouraged to participate in light physical exercise, such as walking, at least 4 times a week.

Proposed mechanism

Although the exact mechanism causing hepatic steatosis is poorly understood, it can be rationalized that hepatic steatosis is a result of net hepatocellular retention of lipids, especially in the form of TAGs (reviewed by Jiang et al., 2013). This imbalance results from abnormalities in hepatic uptake of fatty acids, lipoproteins and glucose, *de novo*

TAG synthesis, TAG degradation and fatty acid β-oxidation and lipoprotein secretion in the form of VLDL (Paredes et al., 2012).

According to the "Two-Hit Hypothesis", a well-studied theory behind the pathogenesis of hepatic steatosis, the "first hit" is the hepatic TAG accumulation leading to steatosis (Feldstein et al., 2004). More specifically, it is when lipid accumulates in the liver due to insulin resistance. Insulin resistance further progresses to hyperinsulinemia and increases lipolysis, which is the breakdown of TAGs to FFA. Lipolysis triggers the release of FFAs from peripheral tissues and results in FFA accumulation in the liver (Mehta et al., 2002). Hepatic TAG accumulation due to hyperinsulinemia occurs because the liver has a reduced ability to re-esterify TAG and package them for transport out of the liver for storage in adipose tissue. Hyperinsulinemia also induces glycolysis and increases the *de novo* synthesis of fatty acids while simultaneously decreasing mitochondrial β-oxidation (Mehta et al., 2002). In the "second hit" of the two hit theory, the mitochondria attempt to oxidize the abundant fatty acids in the liver and thereby cause oxidative stress due to the leakage of reactive oxygen species. Steatosis increases susceptibility of the liver to injury mediated by "second hits", such as inflammatory cytokines and adipokines, mitochondrial dysfunction and oxidative stress, which leads to steatohepatitis and/or fibrosis (Feldstein et al., 2004).

Beyond the "Two-Hit Hypothesis" in the pathogenesis of hepatic steatosis, diet and lifestyle factors also promote the early onset development of NAFLD. Habits associated with poor lifestyle include the frequent consumption of dense calorie foods, rich in both saturated or hydrogenated fats, and high glycemic index carbohydrates, along with consumption of high fructose corn syrup sweetened beverages and carbonated

drinks. This, in combination with the lack of physical activity required to burn the excessive calories that are being consumed, leads to an increase of fat accumulation in adipose tissue, and inappropriately in muscle and liver. This sequence of events leads to the ectopic accumulation of TAGs which develops into insulin resistance, a cascade which has also been referred to as the "overflow hypothesis" (reviewed by Manco et al., 2008).

Metabolic syndrome

The most prevalent risk factor for hepatic steatosis is MetS. According to the International Diabetes Federation, MetS is defined as having $\geq$ 3 of the following conditions: high fasting blood glucose levels (5.6 mmol/L or higher), high blood pressure (120/85 mmHg or higher), high levels of TAGs (1.7 mmol/L or higher), low levels of HDL cholesterol (lower than 1.0 mmol/L in men or 1.3 mmol/L in women) and abdominal obesity (waist circumference >102 cm in men, >88 cm in women)(reviewed by Salazar et al., 2013). It also has been proposed that NAFLD could be considered the hepatic manifestation of MetS (reviewed by Gastaldelli, 2010). MetS in patients is often correlated with the presence of NAFLD. A study done by Fu et al. (2011) showed that in 861 obese children ages 6-16, 68.2% were classified as having NAFLD, and 25.7% as having MetS. Another study done by Valantinas et al. (2011) found that hepatic steatosis outpatients, ages 18-80 (n=798), had a high prevalence of metabolic risk factors such as abdominal obesity (62.4% of men, 89.2% of women), insulin resistance (20.1% of men, 23% of women), hypertension (77.5% of men, 84.0% of women) and dyslipidemia (65.4% in men, 72.4% in women). Due to the fact that NAFLD is a consequence, and a

precursor to many of the health conditions leading to the diagnosis of MetS, several authors have indicated the presentation of NAFLD in MetS patients should be considered.

Obesity

As previously mentioned, a significant number of Canadians, both adults and children, are overweight or obese. Being overweight or obese increases the risk of developing MetS, and it also increases the risk of developing hepatic steatosis. A study done by Ndumele et al. (2012) examined 2,388 adult patients without clinical cardiovascular disease and it showed that steatosis was detected in 32% of the participants with 23% meeting the criteria for MetS and 17% classified as obese. Overall, those with hepatic steatosis were more likely to have MetS and be obese (47% of participants) compared to only 11% of non-obese participants having hepatic steatosis. In a separate study, hepatic steatosis was also evident in 31% of overweight and obese youth, but was not present in the healthy weight youth (Wicklow et al., 2012). The overweight and obese youth with or without hepatic steatosis also displayed lower insulin sensitivity, higher rates of MetS and higher serum glucose after an oral glucose challenge compared to healthy weight adolescents.

In relation to obesity, visceral (abdominal) obesity is an important factor leading to insulin resistance. It is also a main risk factor for the development of NAFLD. It has been shown that abdominal obesity was present in 75% of hepatic steatosis out patients (Valantinas et al., 2011). A study done by Fishbein et al. (2006), reviewed the charts of obese children undergoing evaluation for NAFLD because of hepatomegaly or elevated ALT, a liver enzyme often correlated with liver damage, without obvious etiology. They found that there was a positive correlation between the hepatic fat fraction and total

visceral adipose tissue in the children studied. They also found that elevated serum ALT was associated with a higher hepatic fat fraction and is a risk factor for the development of NAFLD.

As a result of obesity, there is an increase in adipocyte size and adipose cell dysfunction. This consequently leads to an imbalance of pro- and anti-inflammatory factors produced by adipocytes (Fernandez-Riejos et al., 2010). There is also an increase seen in leptin, a homone produced exclusively by adipose tissue (Brennan and Mantzoros, 2006). In adipose tissue from obese individuals, the release of TNF-α stimulates adipocytes to secrete monocyte chemoattractant protein (MCP)-1, leading to macrophage recruitment (reviewed by Hui et al., 2013). Adipose tissue also actively produces anti-inflammatory molecules such as adiponectin (Fernandez-Riejos et al., 2010). Plasma adiponectin concentrations show a negative correlation with body fat mass (Kratz et al., 2008; Stryjecki and Mutch, 2011).

Insulin resistance

Insulin resistance is when the cells in the body become unable to remove glucose from the blood and the body's blood glucose levels continue to rise. Insulin resistance is an important risk factor and determinant of NAFLD. Schwimmer et al. (2003) found that up to 95% of pediatric patients with NAFLD met the criteria for insulin resistance. Another study done by Pacifico et al. (2007) showed that up to 85% of adult subjects (n=1221) with NAFLD, compared to 30% in the control group, were insulin resistant or had abnormal glucose metabolism, such as pre-diabetes or DM2, without even knowing it. Over time the prolonged high levels of insulin suppress the breakdown of TAGs

(lipolysis), and consequently TAGs accumulate in the body (reviewed by Hutfless et al., 2013). Insulin resistance is accompanied by increased circulating insulin levels that, in the presence of increased lipolysis and/or increased fat intake, further promote hepatic TAG synthesis (Gastaldelli et al., 2007). Although the relationship is not dependent, the excess TAGs are stored in visceral fat depots and ectopically, mainly in the liver, and progressively lead to hepatic steatosis.

Dyslipidemia

The presence of dyslipidemia (hypercholesterolemia, hypertriglyceridemia, or both) has been reported in 20% to 80% of cases associated with NAFLD (reviewed by Gaggini et al, 2013). It is also estimated that the prevalence of NAFLD in patients with dyslipidemia attending lipid clinics was about 50% (Assy et al., 2000). In an insulin resistant state, there is an increase in all of the three major sources of free fatty acids (FFA) used for hepatic TAG synthesis. This includes the FFA released via lipolysis from adipose tissues, the fatty acids metabolized from circulating chylomicrons and very low density lipoprotein (VLDL) by lipoprotein lipase (LPL), and fatty acids synthesized *de novo* by lipogenesis (reviewed by Choi and Ginsberg 2011). Insulin modulates lipolysis in adipose tissues and due to insulin resistance there is less inhibition of hormone-sensitive lipase resulting in an increase of serum FFA (reviewed by Jiang et al., 2012). Insulin resistance is also accompanied by an increase in insulin levels such that, in the presence of increased lipolysis and increased fat intake, hepatic TAG synthesis is further enhanced (reviewed by Buzzigold et al., 2013). Under postprandial conditions, in an insulin resistant state, an important source of FFA is the increased spillover from

chylomicrons (reviewed by Gaggini et al., 2013). The increased spillover reflects the inefficiency in dietary fat storage and results in excess FFA. The excess FFAs are taken up by various organs, leading to accumulation of not only hepatic lipids, but also intramyocellular, cardiac and pancreatic ectopic fat (Hwang et al., 2007). The increased ectopic fat accumulation saturates the oxidative capacity of the affected organ, leading to inflammation due to the creation of reactive oxygen species, or the second hit of the hypothesis behind the mechanism of hepatic steatosis (Day and Saskena 2008), as explained in an earlier section.

Hypertension

Blood pressure is a measure of the pressure or force of blood against the walls of the blood vessels. Typically, the blood pressure of the brachial artery is measured and presented in the form xx mmHg/yy mmHg. The top number represents the pressure when the heart contracts and pushes the blood out, or systolic pressure. The bottom number is the lowest pressure when the heart relaxes between beats, also known as diastolic pressure (Public Health Agency of Canada, 2010). A desired blood pressure is between 90-119 mmHg for the systolic pressure, and 60-79 mmHg for diastolic pressure. Pre-hypertension is defined as having blood pressure readings with a systolic pressure from 120 to 139 mmHg or a diastolic pressure from 80 to 89 mmHg. Blood pressure that is consistently more than 140/90 mmHg at the doctor's office, or 135/85 mmHg at home is considered high, and medically is known as hypertension (Public Health Agency of Canada, 2010). The incidence of diabetes and high serum cholesterol increases the risk of developing hypertension, therefore leading to the prevalence of hypertension in MetS

patients (reviewed by Lenfant et al., 2003). Thus, the presence of hypertension is used as a parameter for the diagnosis of MetS, a risk factor for the progression to NAFLD (Liu et al., 2013).

n3 fatty acids

n3 fatty acids are fats that are essential in our diet because the human body cannot synthesize them. Even though we are unable to synthesize them, the human body is able to form longer chain n3 fatty acids from ALA. ALA (C18:3n3) is commonly found in canola and flaxseed oils. Long chain n3 fatty acids commonly found in fish and marine oils, EPA and DHA, can also be formed from ALA, via elongation and desaturation (Figure 1)(Deckelbaum & Torrejon, 2012).

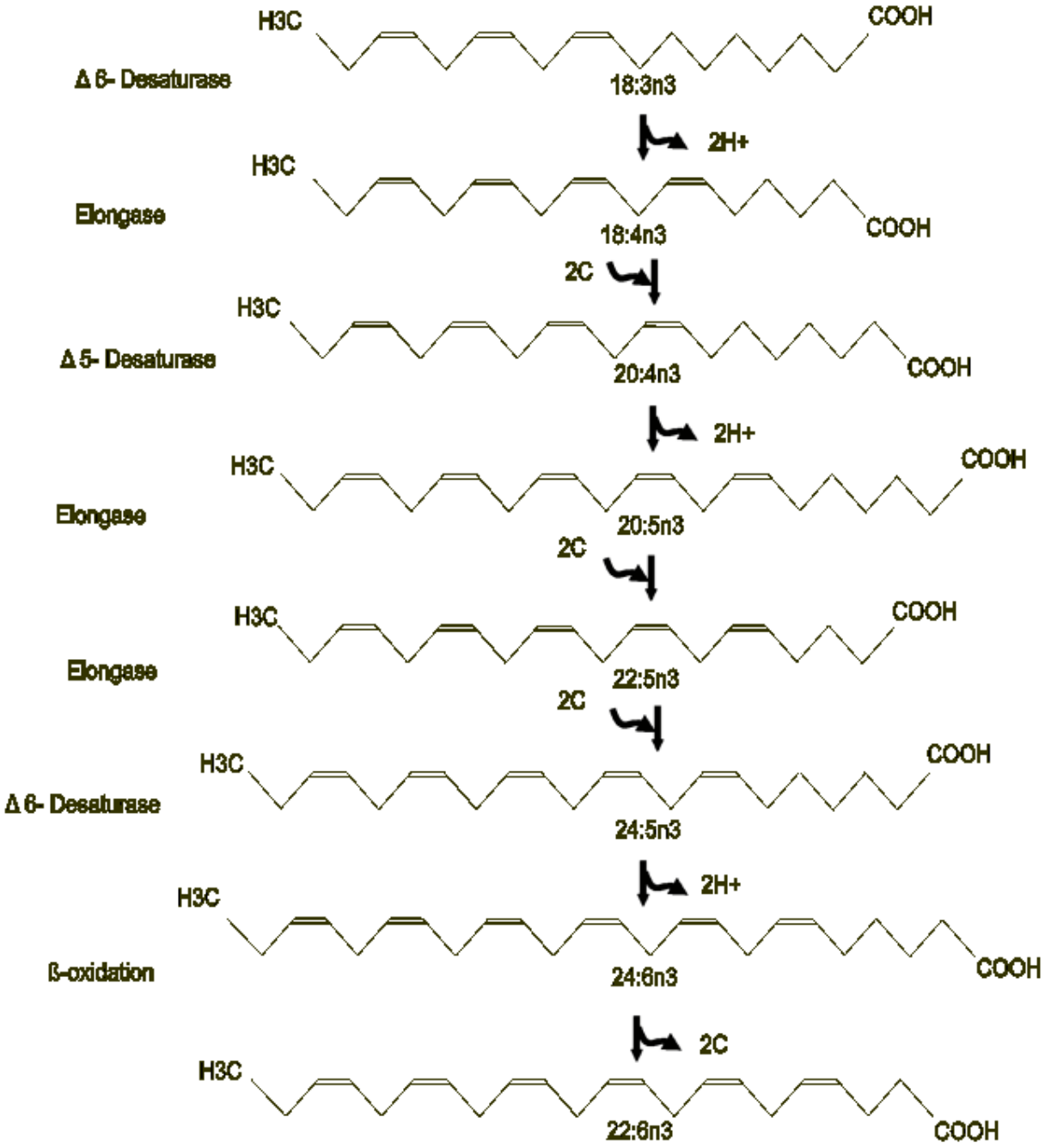


Figure 1: Elongation pathway of ALA to DHA

Effects on hepatic function

Biopsies from people with hepatic steatosis have shown low levels of n3 fatty acids, and this has led to an interest in whether n3 fatty acids might work as a treatment (Gonzales-Periz et al., 2009). There is also evidence that a diet high in n3 fatty acids can decrease the total fat in the liver and improve circulating liver enzymes (e.g. ALT, AST), preventing the progression of hepatic steatosis (reviewed by Bulchandani and Harris, 2006). Previous research shows that fish oil can have beneficial effects on obesity and hepatic steatosis in both humans and in an animal model of obesity (reviewed by Kalupahana 2011; Ahmed & Byrne, 2009). However, studies investigating the effects of dietary plant-based n3 fatty acids on hepatic steatosis and obesity in humans and in animal models for obesity are more limited. The next sections and Tables 1-5 will highlight pertinent published studies of n3 supplementation, hepatic steatosis, adipose function and related parameters.

Fatty acid analyses of biopsies from people with hepatic steatosis have shown low levels of n3 fatty acids (Gonzales-Periz et al., 2009). Araya et al. (2004) found that NAFLD patients had 59-67% less EPA in their livers compared to healthy individuals. NAFLD patients also had 77-86% less DHA in their liver and overall 73-84% less long chain PUFAs in the liver.

There is also evidence that a diet high in n3 fatty acids can decrease the total fat in the liver and reduce circulating liver enzymes, showing a reversal in the progression of hepatic steatosis (reviewed by Di Minno et al., 2012). Zhu et al. (2008) found that supplementation with 2 g of seal oil, an alternative to fish oil, taken 3 times a day by participants was able to reduce NAFLD parameters including significant decreases in the

serum levels of ALT, AST and TAG. They also found that the supplementation was able to reverse lipid accumulation in the liver by 19.7% after the 24 week intervention. Valenzuela et al. (2013) found that daily oral supplementation (by gavage) of n3 at 108 mg/kg/day EPA + 92 mg/kg/day DHA was able to reverse macro and microvesicular steatosis and mild inflammation that was induced by a high fat diet (34.9% [w/w] fat) in C57BL/6J mice. A similar result was seen in Wistar rats that were fed n3 PUFAs as 1.8% (w/w) ALA, 2.6% (w/w) EPA or 2.5% (w/w) DHA in a high fat diet (24% [w/w] fat (Poudyal et al., 2012). Hanke et al. (2013) compared the consumption of high fat diets (31% [w/w fat]) with varying levels of ALA in obese-prone Charles River Sprague Dawley (OP-CD) rats. The group fed the canola/flaxseed oil blend (3:1 canola to flaxseed oil ratio with 6.1% ALA [w/w]) had lower liver lipid concentration compared the other groups fed diets containing minimals amounts of n3. Hanke et al. (2013) also found that the rats fed the canola/flaxseed oil diet had the highest level of n3 in the hepatic phospholipids compared to the other experimental groups.

In Wistar rats fed a high fat diet that was enriched with n3 from chia oil (ALA source), EPA or DHA, Poudyal et al. (2012) found that the serum AST levels were reduced in all three groups. Along with that, Valenzuela et al. (2013) found that daily oral administration of n3 (108 mg/kg/day EPA+ 92 mg/kg/day DHA by gavage) to mice decreased the saturated fatty acid (SFA) content of the liver by 28% and the n6 PUFA content by 23%, all while reducing the n6/n3 PUFA ratio by 70%. It was also shown that n3 supplementation suppressed the circulating levels of IL-6, IL-1β and TNF-α. Riediger et al. (2008) found that fish oil (1.3% [w/w] EPA and 0.9% [w/w] DHA) or flaxseed oil (3% [w/w] ALA) supplementation in a diet containing 19% (w/w) fat decreased total

lipid content in the livers of C57BL/6 mice by 56% and 24%, respectively, and both groups displayed a significantly lower n6:n3 ratio in the liver. The mice fed flax oil had higher levels of DHA and EPA incorporated into phospholipids in the liver compared to the mice fed fish oil. Hanke et al. (2013) found that OP-CD rats fed a diet with a flaxseed/canola oil blend and 6.1% ALA (w/w) had lower circulating ALT and AST than rats fed a diet that contained canola oil alone (2.5% [w/w] ALA). It was also found that the levels of fasting serum haptoglobin, an acute phase protein used as an indicator for chronic inflammation, decreased in the canola/flaxseed oil group compared groups on the other experimental diets.

In relation to hepatic fatty acid oxidation, Rokling-Anderson et al. (2009) found that dietary supplementation of EPA (3.7% [w/w]) and DHA (2.2% [w/w]) was able to increase levels of acetyl CoA carboxylase (ACC) mRNA, an important regulator of fatty acid formation, in the liver. Chechi et al., 2010 also found that a diet containing 5% (w/w) ALA was able to increase the hepatic mRNA levels of peroxisome proliferator-activated receptor alpha (PPARα), a major regulator of lipid metabolism found in the liver, in spontaneously hypertensive (SHR)/NDmcr-cp rats. No differences were seen in the hepatic mRNA levels of sterol regulatory element binding protein (SREBP), a protein that regulates the genes required for glucose metabolism and fatty acid and lipid production (Chechi et al., 2010). Protein levels of PPARα increased in C57BL/6 mice fed a diet containing 4% (w/w) fat with 0.9% (w/w) EPA + DHA compared to a standard chow diet containing 4% (w/w) from fat, while a high fat diet (27.8% [w/w] fat) decreased the amount of PPARα being expressed (Lonzetti Bargut et al., 2014). They also found that SREBP-1 expression increased in mice fed a high fat diet compared to the

standard chow and fish oil diets. Furthermore, there was a decreased in SREBP-1c expression in the high fat fish oil diet (27.8% [w/w] fat and 5.95% [w/w] EPA + DHA) compared to the control high fat diet.

Although positive results have been seen in relation to n3 PUFAs and hepatic fatty acid transcription factors, Gillam et al. (2009) found no differences in the mRNA levels of PPARα and ACC in *fa/fa* Zucker rats. The same was true for Hanke et al. (2013) when they measured hepatic protein levels of PPARα, SREBP, ACC and acetyl-CoA oxidase (ACO), an enzyme involved in fatty acid oxidation.

Hypoxia-inducible protein 2 (HIG2), a hypoxia inducible factor (HIF)-1 target gene, is a novel lipid droplet protein and it works to stimulate intracellular lipid accumulation (reviewed by Okumura, 2011). It's been shown that hepatic overexpression of HIG2 is correlated with an increase in TAG storage in the liver (Mattijssen et al., 2014). Furthermore Mattijssen et al. (2014) found that HIG2 overexpression significantly impaired hepatic TAG secretion. There are currently no published studies of HIG2 gene expression or protein levels in relation to n3 PUFAs.

Effect on adipose function

Baranowski et al. (2012) found that a diet with 10% (w/w) fat supplemented with flaxseed oil that provided 2.8% (w/w) ALA was able to reduce serum leptin, a key hormone in appetite control, and it also reduced the size of the adipocytes by 2-fold in *fa/fa* Zucker rats. There were also differences observed in serum adiponectin related to the genotype of the experimental animals, but none due to the diet itself. Villarroya et al. (2014) found that the supplementation with n3 PUFAs (7.1% [w/w] DHA and 2.2% [w/w]

EPA) in a high fat diet (35% [w/w] fat) showed no significant changes over the course of 5 weeks in serum leptin compared to groups fed either the high fat diet without supplementation or the control low fat diet (3% [w/w] fat). Furthermore, when they continued the feeding study for another 8 weeks, but lowered the n3 PUFA supplementation to 2.4% [w/w] DHA and 0.7% [w/w] EPA), they found that there was an increase in serum leptin in both high fat fed groups and the supplemented groups compared to the low fat control, but there was no difference among the two high fat fed groups. They also found that there was an increase in serum adiponectin in the high fat supplemented group compared to the non-supplemented group and the low fat control in both arms of the study. Lonzetti Bargut et al. (2014) reported that C57JL/6 mice fed a high fat diet (27.8% [w/w] fat) had decreased serum adiponectin compared to the low fat fish oil, standard chow and high fat fish oil groups. Additionally, it was found that consumption of a high fat fish oil diet (5.95% [w/w] EPA + DHA) kept the adiposity of the group similar to low fat controls (4% [w/w] fat). TNF-α and MCP-1, two biomarkers associated with inflammation and adipocyte dysfunction, were elevated in male Wistar rats fed a high fat diet containing 27.8% (w/w) of fat from lard compared to a standard chow control (4% [w/w] fat) (Lonzetti-Bargut et al., 2014). The high fat fish oil diet was able to reduce the circulating levels of TNF-α and MCP-1 compared to the high fat diet without fish oil, but it was unable to reduce them to the level of the low fat control.

Effect on MetS parameters

Obesity

Previous research has shown a correlation between n3 fatty acids and body weight gain. Valenzuela et al. (2013) found that C57BL/6J mice that were provided n3 fatty acids (108 mg/kg/day EPA+ 92 mg/kg/day DHA by gavage) while consuming a high fat diet (39.4% [w/w] fat) gained 41% less weight than their counterparts not consuming the n3 supplement. A high fat diet (21.5% [w/w] fat) that contained 3.7% (w/w) EPA and 2.2% (w/w) DHA decreased the weight of the peri-renal adipose tissue by 51%, epidydimal fat by 31% and mesenteric adipose weight by 35% compared to the Wistar rats fed the lard-based control diet containing 21.5% [w/w] from fat (Rokling-Anderson et al., 2009).

Fibroblast growth factor (FGF)-21 is a metabolic hormone predominately produced in the liver (reviewed by Woo et al., 2013). There is elevated circulating FGF-21 in humans with obesity, MetS, DM2, NAFLD and coronary heart disease (reviewed by Woo et al., 2013). Furthermore, an increase of FGF-21 mRNA expression has been shown during the conversion of human pre-adipocytes to mature adipocytes (reviewed by Hui et al., 2013). Previous research has showned that the administration of recombinant FGF-21 has shown various metabolic benefits on insulin sensitivity, blood glucose, lipid profile and body weight in obese mice and diabetic monkeys without mitogenic or other side effects (reviewed by Woo et al., 2013).

In the past it has been shown that LC-PUFAs may counteract the development of obesity and decrease MetS in rodents (Villarroya et al., 2014). Villarroya et al. (2014) tested to see if the increase expression of FGF-21 induced by n3 PUFA supplementation

could be the mechanistic link between n3 PUFAs and the potential to attenuate MetS parameters in an obesity model. In the first part of their study they fed 8 week old C57BL/6J mice a high fat diet containing 35% (w/w) corn oil or a high fat diet with n3 PUFAs (7.1% DHA [w/w] and 2.2% EPA [w/w]) or a control low fat diet containing 3% (w/w) corn oil. The mice were fed these experimental diets over the course of 5 weeks. They found that there was an increase in plasma FGF-21 in the supplemented group compared to the non-supplemented group and control with no differences in mRNA levels of FGF-21. In a second follow up study, they extended the treatment period for another 8 weeks on the same mice, this time decreasing the n3 PUFAs supplementation to 2.4% (w/w) DHA and 0.7% (w/w) EPA. They found that there was still an increase in plasma FGF-21 in the supplemented group compared to the non-supplemented and control groups with no further differences seen in hepatic FGF-21 mRNA levels.

Insulin resistance

There is evidence to show that dietary n3 fatty acids can decrease circulating insulin and lipids and improve glucose tolerance leading to an improvement in health status. Valenzuela et al. (2013) subjected C57BL/6J mice for 12 weeks to a high fat diet (34.9% [w/w] fat) and reported that n3 supplementation (108 mg/kg/day EPA + 92 mg/kg/day DHA by gavage) was able to normalize serum glucose and insulin levels. Rokling-Anderson et al. (2009) found that supplementing a high fat diet (21.5% w/w fat) with 3.7% (w/w) EPA and 2.2% (w/w) DHA for 7 weeks was able to reduce serum insulin concentrations by 72% in Wistar rats compared to the lard control diet. Hanke et al. (2013) found that a high fat diet (31% w/w fat) containing 6.1% ALA (w/w) was able

to reduce fasting serum glucose in OP-CD rats, compared to the rats that were on a diet high in saturated fats. Gillam et al. (2009) was also able to show that a diet containing 10% (w/w) fat with 0.8% EPA (w/w) and 0.9% DHA (w/w) from menhaden oil was able to reduce circulating insulin in *fa/fa* Zucker rats. Chechi et al. (2010) was able to show that a diet supplemented with 5% (w/w) ALA from flaxseed oil was able to do the same in SHR/NDmcr-cp rats. Poudyal et al. (2012) found that n3 supplementation as 1.8% (w/w) ALA, 2.6% (w/w) EPA or 2.5% (w/w) DHA in a high fat diet (24% [w/w] fat was able to reduce circulating insulin levels and improve their response in an oral glucose tolerance test. Villarroya et al. (2014) found that the supplementation of 9.2% (w/w) n3 PUFAs (7.1% DHA [w/w], 2.2% EPA [w/w]) was able to decrease serum insulin in C57BL/6J mice, with no effects on serum glucose. The same was seen when they continued the supplementation, but decreased the amount of n3 PUFAs being supplemented to 2.4% (w/w) DHA and 0.74% (w/w) EPA. Samane et al. (2009), found that the addition of fish oil (0.96% [w/w] EPA and 0.65% [w/w] DHA) in a high fat diet (39.6% [w/w] fat) was able to decrease serum insulin of Male Wistar rats compared to those fed the high fat diet without fish oil and to similar levels as the control group being fed a standard chow containing 5.6% (w/w) fat. The fish oil fed group also had lower circuling insulin during an intraperitoneal glucose tolerance test (IPGTT) than the group fed a high fat diet without fish oil, with similar values as the standard chow control.

Protein tyrosine phosphatase 1B (PTP1B) plays an important role in the negative regulation of insulin signal transduction pathway and has emerged as a novel therapeutic strategy for the treatment of DM2 (reviewed by Tamrakar et al., 2014). It's been shown that PTP1B inhibitors enhance the sensitivity of insulin receptors by increasing the

phosphorylation of the insulin receptor and its substrates. Currently, many patent applications, clinical trials and drug validation experiments have involved PTP1B inhibitors individually or in combination with other interventions, but there is no published literature on PTP1B inhibition and n3 PUFAs.

Resistin is a peptide hormone, derived from adipocytes and immune cells whose physiological properties have been debatably related to obesity and DM2 (reviewed by McTeran et al., 2006). It has been established that adipose tissue over-production is responsible for increased serum resistin levels; however, there is currently controversy in the literature whether or not human resistin is secreted from the adipocytes, preadipocytes or macrophages (reviewed by McTeran et al., 2006). It's been shown that the administration of resistin *in vivo* and transgenic overexpression of resistin induces insulin resistance by increasing hepatic glucose production in Sprague Dawley rats; thus resistin might link obesity and insulin resistance in a rat model. Lonzetti Bargut et al., (2014) found that the consumption of a high fat diet increased circulating resistin concentrations, while a high fat diet supplemented with fish oil kept resistin concentrations similar to a low fat diet control in C57BL/6 mice.

Dyslipidemia and hypertension

Chechi et al. (2010) found that there was a decrease in TAG in SHR/NDmcp-cp rats fed a diet supplemented with 3.4% ALA (w/w) from flax oil, versus the animals on the lard control diet, and a significant decrease (67%) in serum cholesterol in the flaxseed oil-fed group compared to the lard-fed control group. Yang et al. (2009) also found that there was a decrease in serum TAG in golden Syrian hamsters fed a diet that contained 5.3% ALA (w/w) from flaxseed oil compared to the hamsters fed a diet that contained

coconut oil, or butter as the fat source and the control group, respectively. There was also a decrease in serum cholesterol in the flaxseed oil diet group compared to the other two groups along with the control group. It was also shown that n3 fatty acid supplementation with ALA, EPA or DHA could reduce serum FFA, TAG and cholesterol in Wistar rats fed a high fat diet (24% [w/w]) (Poudyal et al., 2012). The effect was more dramatic in the high fat diet, although supplementation of EPA and DHA was shown to decreased serum TAG and cholesterol in lower fat (0.81% [w/w]) diets as well. Hanke et al. (2013) found that OP-CD rats on a high fat diet (31% [w/w]) that contained 6.1% ALA (w/w) had lower fasting serum TAG compared to groups that were fed a diet high in oleic oil or lard. Valenzuela et al. (2013) also found that n3 supplementation with EPA and DHA was able to decrease the serum TAG by 11% in C57BL/6J mice. Riediger et al. (2007) found that in the same model, 3% (w/w) from ALA was more effective for lowering serum cholesterol levels in mice than fish oil or safflower oil-based diets that containined 2% (w/w) cholesterol and 0.2% (w/w) cholic acid. Lonzetti Bargut et al. (2014) reported that a high fat diet (27.8% [w/w] fat) containing 5.95% (w/w) EPA + DHA was able to reduce serum TAG and total cholesterol compared to the low fat controls.

There are no studies that look at the direct relationship between n3 PUFAs and hypertension although there is an increase the risk of developing hypertension with higher incidences of diabetes and high serum cholesterol (Kaplan, 2002).

Table 1: Effects of n3 fatty acids on serum ALT, AST and haptoglobin

Reference	Model and diet	Overall amount of n3 in diet (w/w) & type consumed	Total % fat in diet (w/w)	Length of study	Significant effects of n3 compared to control animals		
					ALT	**AST**	**Haptoglobin**
Valenzuela et al., 2013	Weaning male C57BL/6J mice divided into 4 groups: control (CTL), CTL+n3 supplementation, high fat diet (HFD) or HFD+n3	CTL: <1% ALA HFD: <1% ALA * Daily oral supplementation of 108 mg/kg/day EPA+ 92 mg/kg/day DHA was given to the experimental groups	4.3% in CTL 34.9% in HFD	12 weeks	ND	ND	DNM
Poudyal et al., 2012	9-10 week old Wistar rats divided into 8 diet groups: corn starch (C), C+chia oil (CA), C+EPA oil (CE), C+DHA oil (CD), high carbohydrate+high fat (H), H+chia oil (HA), H+EPA oil (HE), H+DHA (HD)	C: <0.1% n3 H: 2.4% n3 CA: 1.8% ALA CE: 2.6% EPA CD: 2.5% DHA HA: 4.2% ALA HE: 5.0% EPA	C: 0.8% H: 24% CA: 3.8%* CE: 3.8%* CD: 3.8* HA: 27%* HE: 27%*	16 weeks	↑ in CE and CD	↓ in HA, HE and HD ↑ in CE and CD	DNM

		HD: 4.9% DHA *The n3 was added into the diet 8 weeks after the start of the C or H diet	HD: 27% *				
Baranowski et al., 2012	17 week old male lean and *fa/fa* Zucker rats divided into 3 diet groups: *fa/fa* control (faCTL), lean control (lnCLTL) and *fa/fa* flax (faFLAX)	faFLAX: 2.8% ALA faCTL: <1% ALA lnCTL: <1% ALA	8.5% in all diets	8 weeks	DMN	DNM	↑ in faFLAX
Hanke et al., 2013	6 week old OP-CD rats divided into 7 groups: high oleic canola (HOC), conventional canola (Ca), conventional canola/flax oil blend (Ca/F), high linoleic safflower (SF), lard/soybean oil blend (L), and a weight matched group on a lard/soybean oil blend (WM)	HOC: <1% ALA C: 2.5% ALA C/F: 6.1% ALA SF: <1% ALA SB: 2.8% ALA L : <1% ALA WM : <1% ALA	31%	12 weeks	↑ in Ca and HOC	↑ in Ca	↓ in Ca/F, L, and WM

- ND = no difference
- DNM = did not measure

Table 2: Effects of n3 fatty acids on molecular markers of hepatic fatty acid oxidation and hepatic fatty acid synthesis

Reference	Model and diet	Overall amount of n3 in diet (w/w) & type consumed	Total % fat in diet (w/w)	Length of study	Significant effects of n3 compared to control animals			
					PPARα	ACO	SREBP-1	ACC
Rokling-Anderson et al., 2009	7 week old Wistar rats divided into 2 diet groups: lard (L) or n3 from EPA/DHA (n3)	L: <0.1% ALA n3: 3.7% EPA, 2.2% DHA	21.5%	7 weeks	DNM	DNM	DNM	↑ in the n3 group
Gillam et al., 2009	6 week old male *fa/fa* Zucker rats divided into 3 diet groups: flaxseed oil (FXO), menhaden oil (MO) or safflower oil (SF)	FXO: 3.5% ALA MO: 0.8% EPA, 0.9% DHA (1.7 % total) SF: <1% ALA	10%	9 weeks	ND	ND	DNM	DNM
Chechi et al., 2010	7 week old obese and lean SHR/NDmcr-cp rats divided into 4 groups: obese fed flax oil (FO), lean fed flax oil (FL), obese fed lard (LO) and lean fed lard (LL)	FO: 5% ALA FL: 0.8% ALA LO: 5% ALA LL: 0.8% ALA	15%	4 weeks	↑ in FO vs FL	DNM	ND	DNM

Hanke et al., 2013	6 week old OP-CD rats divided into 7 groups: high oleic canola (HOC), conventional canola (C), conventional canola/flax oil blend (C/F), high linoleic safflower (SF), lard/soybean oil blend (L), and a weight matched group on a lard/soybean oil blend (WM)	HOC: <1% ALA C : 2.5% ALA C/F: 6.1% ALA SF: <1% ALA SB: 2.8% ALA L : <1% ALA WM : <1% ALA	31%	12 weeks	ND	ND	ND	ND
Lonzetti-Bargut et al., 2014	12 week old male C57BL/6 mice fed either a standard chow diet (SC), low fat fish oil diet (FO), a high fat diet (HF) or a high-fish oil (HFO) diet	SC: <1% n3 FO: 0.9% EPA+DHA HF: <1% n3 HFO: 5.95% EPA+ DHA	SC: 4% FO: 4% HF: 27.8% HFO: 27.8%	8 weeks	↑ in FO compared to SC and HFO ↓ in HF	DNM	↑ in HF compared to SC and FO ↓ in HFO compared to HF	DNM

- ND = no difference
- DNM = did not measure

Table 3: Effects of n3 fatty acids on fasting glucose, insulin and glucose tolerance

Reference	Model and diet	Overall amount of n3 in diet (w/w) & type consumed	Total % fat in diet (w/w)	Length of study	Significant effects of n3 compared to control animals		
					Glucose	Insulin	OGTT
Rokling-Anderson et al., 2009	7 week old Wistar rats divided into 2 diet groups: lard (L) or n3 from EPA/DHA (n3)	L: <0.1% ALA n3: 3.7% EPA, 2.2% DHA	21.5%	7 weeks	ND	↓ in n3	DNM
Gillam et al., 2009	6 week old male *fa/fa* Zucker rats divided into 3 diet groups: flaxseed oil (FXO), menhaden oil (MO) or safflower oil (SO)	FXO: 3.5% ALA MO: 0.8% EPA, 0.9% DHA (1.7 % total) SF: <1% ALA	10%	9 weeks	ND	↓ fasting serum insulin in SO	No significant difference in OGTT
Chechi et al., 2010	7 week old obese and lean SHR/NDmcr-cp rats divided into 4 groups: obese fed flax oil (FO), lean fed flax oil (FL), obese fed lard (LO) and lean fed lard (LL)	FO: 3.4% ALA FL: 0.5% ALA LO: 3.4% ALA LL: 0.5% ALA	15%	4 weeks	ND	↓ serum insulin in FL compared to FO	DNM

Valenzuela et al., 2013	Weaning male C57BL/6J mice divided into 4 groups: control (CTL), CTL+n3 supplementation, high fat diet (HFD) or HFD+n3	CTL: <1% n3 HFD: <1% n3 * Daily oral supplementation of 108 mg/kg/day EPA+ 92 mg/kg/day DHA was given to the experimental group	4.3% in CTL 34.9% in HFD	12 weeks	↑ 70% in HFD and normalized after n3 supplementation	↑ 200% in HFD and normalized after n3 supplementation	DNM
Poudyal et al., 2012	9-10 week old Wistar rats divided into 8 diet groups: corn starch (C), C+chia oil (CA), C+EPA oil (CE), C+DHA oil (CD), high carbohydrate+high fat (H), H+chia oil (HA), H+EPA oil (HE), H+DHA (HD)	C: <0.1% n3 H: 2.4% n3 CA: 1.8% ALA CE: 2.6% EPA CD: 2.5% DHA HA: 4.2% ALA HE: 5.0% EPA HD: 4.9% DHA	C: 0.8% H: 24% CA: 3.8%* CE: 3.8%* CD: 3.8* HA: 27%* HE: 27%* HD: 27% * *The n3 was added into the	16 weeks	ND	400% ↑ in H versus C rats ↓ in HA, HE and HD rats	OGTT only improved in the HA rats

			diet 8 weeks after the start of the C or H diet				
Hanke et al., 2013	6 week old OP-CD rats divided into 7 groups: high oleic canola (HOC), conventional canola (C), conventional canola/flax oil blend (C/F), high linoleic safflower (SF), lard/soybean oil blend (L), and a weight matched group on a lard/soybean oil blend (WM)	HOC: <1% ALA Ca : 2.5% ALA C/F: 6.1% ALA SF: <1% ALA SB: 2.8% ALA L : <1% ALA WM : <1% ALA	31%	12 weeks	↓ in HOC, C, C/F and SF compared to L and WM	ND	DNM
Villarroya et al., 2014 (1)	8 week old C57BL/6J mice were fed one of 3 dietary groups: a high carbohydrate low fat control (C), corn-oil based high-fat diet (cHF) or a cHF diet, supplemented with LC PUFAs (cHF+F) 2 weeks into the study	C: <1% n3 cHF: <1% n3 cHF+F: 7.1% DHA, 2.2% EPA (9.3% total)	C: 3% cHF: 35% cHF+F: 35%	5 weeks	ND	↓ in cHF+F compared to C and cHF	DMN

Villarroya et al., 2014 (2)	16 week old C57BL/6J mice were fed one of 3 dietary groups: a high carbohydrate low fat control (C), corn-oil based high-fat diet (cHF) or a cHF diet, supplemented with LC PUFAs (cHF+F).	C: <1% n3 cHF: <1% n3 cHF+F: 2.4% DHA, 0.74 % EPA (3.14% total)	C: 3% cHF: 35% cHF+F: 35%	8 weeks	ND	↑ in cHF and ↓ in cHF+F compared to control	DNM
Lonzetti-Bargut et al., 2014	12 week old male C57BL/6 mice fed either a standard chow diet (SC), low fat fish oil diet (FO), a high fat diet (HF) or a high-fish oil (HFO) diet	SC: <1% n3 FO: 0.9% EPA+DHA HF: <1% n3 HFO: 5.95% EPA+ DHA	SC: 4% FO: 4% HF: 27.8% HFO: 27.8%	8 weeks	ND between SC and FO. HFO had higher fasting glucose than SC and FO, but not as high as HF	↑ in HF compared to SC and FO. HFO were also higher than SC and FO, but not as high as HF	Improved in HFO compared to HF
Samane et al., 2009	Male Wistar rats without a specified age fed either a standard chow control (SC), high fat/high sucrose diet (HFHS), HFHS with fish oil (FO) or HFHS with argan oil	SC: <1% ALA HFHS: <1% ALA FO: 0.96% EPA, 0.65% DHA (1.65% total n3) AO: <1% ALA	SC: 5.6% HFHS: 39.6% FO: 39.6% AO: 39.6%	4 weeks	↑ in HFHS compared to chow	↓ in FO compared to HFHS	FO was similar to SC, which is lower than HFHS and AO* *Measured by IPGTT

- ND = no difference
- DNM = did not measure

Table 4: Efects of n3 fatty acids on leptin, adiponectin and adiposity

Reference	Model and diet	Overall amount of n3 in diet (w/w) & type consumed	Total % fat in diet (w/w)	Length of study	Significant effects of n3 compared to control animals		
					Leptin	Adiponectin	Adiposity
Baranowski et al., 2012	17 week old male lean and *fa/fa* Zucker rats divided up into 3 diet groups; *fa/fa* control (faCTL), lean control (lnCLTL) and *fa/fa* flax (faFLAX)	faFLAX: 2.8% ALA faCTL: <1% ALA lnCTL: <1% ALA	8.5%	8 weeks	Difference seen between genotypes (↑ in faCTL compared to lnCTL); ↓ in faFLAX	Difference observed due to genotype (↓ in adipose, ↑ in serum of *fa/fa*) but not due to diet	2× greater adipocyte size in faCTL compared to lnCTL; ↓ in faFLAX
Rokling-Anderson et al., 2009	7 week old Wistar rats divided into 2 diet groups: lard (L) or n3 from EPA/DHA (n3)	L: <0.1% ALA n3: 3.7% EPA, 2.2% DHA	21.5%	7 weeks	ND	ND	n3 had ↓ 51% in perirenal adipose weight ↓ 31% epidydimal fat adipose weight and ↓ 35% mesenteric adipose weight compared to L

Gillam et al., 2009	6 week old male *fa/fa* Zucker rats divided into 3 diet groups: flaxseed oil (FXO), menhaden oil (MO) or safflower oil (SO)	FXO: 3.5% ALA MO: 0.8% EPA, 0.9% DHA (1.7 % total) SF: <1% ALA	10%	9 weeks	27 fold difference between genotypes, regardless of diet ND respective of diet	DNM	ND
Villarroya et al., 2014 (1)	8 week old C57BL/6J mice were fed one of 3 dietary groups: a high carbohydrate low fat control (C), corn-oil based high-fat diet (cHF) or a cHF diet, supplemented with LC PUFAs (cHF+F) 2 weeks into the study	C: <1% n3 cHF: <1% n3 cHF+F: 7.1% DHA, 2.2% EPA (9.3% total)	C: 3% cHF: 35% cHF+F: 35%	5 weeks	ND	↑ In cHF+F compared to C and CHF	↑ WAT weight in cHF compared to cHF+F and C. C and cHF+F were similar
Villarroya et al., 2014 (2)	16 week old C57BL/6J mice were fed one of 3 dietary groups: a high carbohydrate low fat control (C), corn-oil based high-fat diet (cHF) or a cHF diet, supplemented with LC PUFAs (cHF+F).	C: <1% n3 cHF: <1% n3 cHF+F: 2.4% DHA, 0.74 % EPA (3.14% total)	C: 3% cHF: 35% cHF+F: 35%	8 weeks	↑ in cHF and cHP+F compaed to control, no differences among the two	↑ in cHF+F compared to C and cHF	↑ in cHF+F compared to C, but not as high as cHF

Lonzetti-Bargut et al., 2014	12 week old male C57BL/6 mice fed either a standard chow diet (SC), low fat fish oil diet (FO), a high fat diet (HF) or a high-fish oil (HFO) diet	SC: <1% n3 FO: 0.9% EPA+DHA HF: <1% n3 HFO: 5.95% EPA+ DHA	SC: 4% FO: 4% HF: 27.8% HFO: 27.8%	8 weeks	DNM	↓ in HF compared to other groups. ND between HFO, FO and SC.	↑ in HF compared to the other groups. ND between HFO, FO and SC

- ND = no difference
- DNM = did not measure

Table 5: Effects of n3 fatty acids on circulating free fatty acids, TAG and cholesterol

Reference	Model and diet	Overall amount of n3 in diet (w/w) & type consumed	Total % fat in diet (w/w)	Length of study	Significant effects of n3 compared to control animals		
					FFA	TAG	Cholesterol
Valenzuela et al., 2013	Weaning male C57BL/6J mice divided into 4 groups: control (CTL), CTL+ n3 supplementation, high fat diet (HFD) or HFD+n3	CTL: <1% n3 HFD: <1 % n3 * Daily oral supplementation of 108 mg/kg/day EPA+ 92 mg/kg/day DHA was given to the experimental group	4.3% in CTL 34.9% in HFD	12 weeks	DNM	↓ 11% in n3 group	ND
Gillam et al., 2009	6 week old male *fa/fa* Zucker rats divided into 3 diet groups: flaxseed oil (FXO), menhaden oil (MO) or safflower oil (SO)	FXO: 3.5% ALA MO: 0.8% EPA, 0.9% DHA (1.7 % total) SF: <1% ALA	10%	9 weeks	↓ in MO	ND	DMN

Chechi et al., 2010	7 week old obese and lean SHR/NDmcr-cp rats divided into 4 groups: obese fed flax oil (FO), lean fed flax oil (FL), obese fed lard (LO) and lean fed lard (LL)	FO: 5% ALA FL: 0.8% ALA LO: 5% ALA LL: 0.8% ALA	15%	4 weeks	DNM	↓ in FX vs FL and in FO vs FL	↓ LDL-cholesterol in FL vs LL
Yang et al., 2009	5 week old male Golden Syrian hamsters divided into 4 groups: CTL, coconut oil (CO), butter (BU) and flaxseed oil (FX)	CTL: <1% n3 CO: <1% n3 BU: <1% n3 FX: 5.3% ALA	5% in CTL 12% in CO, BU and FX	6 weeks	DNM	↓ in FX vs CO and BU	↓ in FX vs CO and BU
Poudyal et al. 2012	9-10 week old Wistar rats divided into 8 diet groups: corn starch (C), C+chia oil (CA), C+EPA oil (CE), C+DHA oil (CD), high carbohydrate+high fat (H), H+chia oil (HA), H+EPA oil (HE), H+DHA (HD)	C: <0.1% n3 H: 2.4% n3 CA: 1.8% ALA CE: 2.6% EPA CD: 2.5% DHA HA: 4.2% ALA HE: 5.0% EPA HD: 4.9% DHA	C: 0.8% H: 24% CA: 3.8%* CE: 3.8%* CD: 3.8* HA: 27%* HE: 27%* HD: 27% *	16 weeks	↓ in HA, HE, and HD	↓ in HA, HE, and HD, CE and CD	↓ in HA, HE, and HD, CE and CD

			*The n3 added into the diet 8 weeks after the start of the C or H diet				
Hanke et al., 2013	6 week old OP-CD rats divided into 7 groups: high oleic canola (HOC), conventional canola (C), conventional canola/flax oil blend (C/F), high linoleic safflower (SF), lard/soybean oil blend (L), and a weight matched group on a lard/soybean oil blend (WM)	HOC: <1% ALA C: 2.5% ALA C/F: 6.1% ALA SF: <1% ALA SB: 2.8% ALA L : <1% ALA WM : <1% ALA	31%	12 weeks	ND	↓ in C/F, SF and SB compared to HOC, L and WM	ND
Riediger et al., 2007	male C57BL/6 mice divided into 3 diet groups: fish oil based (FO) flax seed oil based (FX), and safflower oil based (SF) *all diets contained 2% w/w cholesterol and 0.2% w/w cholic acid	FO: 0.4% ALA, 1.3% EPA and 0.9% DHA FX: 3% ALA SF: 0.3% ALA	19%	16 weeks	DNM	ND between groups	Less increase in FX vs. FO and SO

Lonzetti-Bargut et al., 2014	12 week old male C57BL/6 mice fed either a standard chow diet (SC), low fat fish oil diet (FO), a high fat diet (HF) or a high-fish oil (HFO) diet	SC: <1% n3 FO: 0.9% EPA+DHA HF: <1% n3 HFO: 5.95% EPA+ DHA	SC: 4% FO: 4% HF: 27.8% HFO: 27.8%	8 weeks	DNM	↑ in HF compared to SC and FO. ↓ in HFO compared to the other groups	↑ in total cholesterol in HF while HFO had the lowest.

- ND = no difference
- DNM = did not measure

fa/fa Zucker rat model

Hepatic Steatosis

Serkova et al. (2005) has reported that there is a significant difference in hepatic FA and TAG concentration in *fa/fa* Zucker rats versus lean Zucker rats fed the same diet. It was also reported that the ratios of PUFA to MUFA were also significantly lower in obese rats compared to lean rats and similar in the liver, whereas the blood levels of PUFAs were decreased and MUFA increased (Serkova et al., 2005). These are all indicators useful for tracking the progression of NAFLD regression.

Metabolic Syndrome

Obesity

The *fa/fa* Zucker rat is the most widely used rat model for genetic obesity (reviewed by Greenwood et al., 1985). The *fa/fa* rats become noticeably obese by 3-5 weeks of age and by 14 weeks they will have 40% greater body fat than their lean counterparts (Zucker and Antoniades, 1972). The *fa/fa* Zucker rats are hyperphagic compared to their lean counter parts, particularly during periods of rapid growth (first 16 weeks of life) (reviewed by Kava et al., 1990). They also have enhanced lipoprotein lipase activity, which is significantly correlated with enhanced TAG uptake by adipose tissue (Maggio and Greenwood, 1982).

Insulin resistance

A significant difference in fasting serum glucose is not often seen in the *fa/fa* Zucker versus their lean counterparts. However, the rate at which glucose drops in the

blood following a glucose load is significantly slower than in a lean Zucker, showing impaired glucose tolerance (Zucker and Antoniades, 1972). Hyperinsulinemia is observed in *fa/fa* Zucker rats as early as 4 weeks of age, reaching a plateau at 15 weeks of age (Zucker and Antoniades, 1972). The skeletal muscle of *fa/fa* Zucker rats is highly insulin resistant with depressed basal and insulin-stimulated transport (reviewed by Kava et al., 1990). Additionally, the obese Zucker rat exhibits severe hepatic as well as peripheral insulin resistance (Terralaz et al., 1986).

Dyslipidemia

Dyslipidemia is often a consequence of insulin resistance and *fa/fa* Zucker rats have elevated serum lipid concentrations versus lean Zucker rats (Serkova et al., 2005). Although a difference in serum TAG is seen when directly comparing a *fa/fa* Zucker rat to a lean Zucker rat on the same diet, total cholesterol was not elevated in the liver or the serum of the *fa/fa* Zucker rats in the same study.

Hypertension

Insulin resistance is often reported in patients with high blood pressure and it has been proposed that insulin resistance is an important pathogenic determinant of hypertension in obese patients (Kurtz et al., 1989). The *fa/fa* Zucker rats have been shown to develop insulin resistance at an early age (reviewed by Kava et al., 1990) and mean arterial pressure of *fa/fa* Zucker rats is significantly higher than lean Zucker rats while controlling for feed intake (Kurtz et al., 1989).

Proposed Mechanism for Reduced Hepatic Steatosis with Dietary n3 Fatty Acids

Dietary n3 fatty acids regulate hepatic gene expression by targeting two major transcriptional regulatory networks, PPARα and SREBP-1. Through the expression, or lack of expression, of these proteins, they are able to control glycolysis, *de novo* lipogenesis and fatty acid elongation, desaturation and oxidation (reviewed by Di Minno et al., 2012). Two general mechanisms characterize fatty acid control of these transcription factors:

1. Fatty acids bind to and control the activity of specific nuclear receptors. Specifically, they act like hydrophobic hormones to regulate nuclear receptor function. Non-esterified fatty acids bind to PPARα, which functions as a fatty acid-regulated transcription factor *in vivo*. Fatty acids control the nuclear abundance of SREBP-1, amongst other transcription factors.
2. PPARα and SREBP-1 regulate multiple enzymes involved with hepatic and lipid metabolism. PUFA-induced activation of PPARα enhances fatty acid oxidation, while PUFA-dependent suppression of SREBP-1 results in the inhibition of *de novo* synthesis of lipids, including PUFAs. This shift alters hepatic VLDL composition, which in turn increases hepatic lipids. The effects are seen on lipid synthesis in the liver, but not necessarily in other tissues. ACC and ACO can be used as downstream indicators of fatty acid synthesis and oxidation, respectively.

A strong association exists between NAFLD and DM2, with upwards of 70% of patients with DM2 having NAFLD (Schwimmer et al, 2003; Pacifico et al., 2007; Hui et al., 2013). In addition to hepatic insulin resistance, NAFLD is associated with a defect in

insulin-mediated suppression of lipolysis, in keeping with insulin resistance in adipose tissue (reviewed by Hui et al., 2013). These findings suggest that insulin resistance might have an intrinsic defect in NALFD similar to that DM2, leading to a decrease in insulin responsiveness at the levels of the adipocytes which potentially contributes to hepatic steatosis through excess free fatty acid influx to the liver (reviewed by Byrne, 2012).

Protein levels of PTP-1B and FGF-21 have been associated with improvements in insulin resistance (reviewed by Tamrakar et al., 2014; reviewed by Woo et al., 2013). It's been shown that PTP1B inhibitors enhance the sensitivity of insulin receptors by increasing the phosphorylation of the insulin receptor and its substrates. Administration of recombinant FGF-21 has also been shown to have various metabolic benefits on insulin sensitivity, blood glucose, lipid profile and body weight, which are all factors related to the progression of hepatic steatosis (reviewed by Woo et al., 2013). Thus, the inhibition of PTP1B or the increased expression of FGF-21 in response to n3 PUFAs could be a contributing mechanism to the attenuation of hepatic steatosis.

HIG2 is a novel lipid droplet protein that works to stimulate intracellular lipid accumulation (reviewed by Okumura, 2011). It's been shown that hepatic over expression of HIG2 is correlated with an increase in TAG storage in the liver (Mattijssen et al., 2014). The supplementation of n3 PUFAs could potentially decrease the expression of HIG2, delaying the onset or progression of hepatic steatosis. There is no current published literature regarding HIG2 and n3 PUFAs.

STUDY RATIONALE

Although there is some research evidence that fish oil can have beneficial effects on hepatic steatosis, adipose function and MetS parameters, studies investigating the effects of dietary plant-based n3 fatty acids on obesity and hepatic steatosis are more limited. Furthermore, a direct comparison of EPA versus DHA has not been done. This would be the first study to directly compare plant-based ALA in flaxseed oil and marine based EPA and DHA for their effects on specific metabolic parameters in a *fa/fa* Zucker rat model. It will also be the first study to compare high purity EPA with high purity DHA.

HYPOTHESIS

It is hypothesized that dietary intervention with n3 fatty acids from plant-based sources (ALA) will be as effective as dietary intervention with n3 fatty acids from marine sources (EPA and DHA) for reducing hepatic steatosis, and improving adipose function and MetS parameters in *fa/fa* Zucker rats. It is also hypothesized that ALA will be as effective as EPA or DHA for increasing hepatic fatty acid oxidation and decreasing hepatic fatty acid synthesis.

OBJECTIVES

To compare the effects of dietary intervention with a canola/flaxseed oil blend (containing ALA) versus EPA and versus DHA while maintaining similar proportions of SFA, MUFA and PUFA on hepatic steatosis parameters, adipose function and MetS parameters in *fa/fa* Zucker rats. The following specific objectives are defined:

1. To determine the effects of various dietary PUFA compositions on hepatic steatosis and associated parameters by measuring:

a) Total liver lipid concentration

b) Hepatic function (serum AST, ALT and haptoglobin)

c) Liver histology (size and number of lipid droplets)

d) Hepatic fatty acid composition of TAG and phospholipid (PL)

e) Markers of hepatic fatty acid oxidation (PPARα, pPPARα and ACO protein levels)

f) Markers of hepatic fatty acid synthesis (SREBP-1, ACC and pACC protein levels)

g) Markers of hepatic lipid uptake (HIG2 protein levels)

2. To examine the effects of an 8 week dietary intervention with different n3 PUFA sources in *fa/fa* Zucker rats on adipose function by measuring:

a) Adiposity (fat pad mass and fat pad/body weight ratios, body composition)

b) Adipocyte size (morphometry)

c) Pro-inflammatory adipokines (serum MCP-1, leptin and TNF-α)

d) Anti-inflammatory adipokines (serum resistin and adiponectin)

3. To determine the effects of an 8 week dietary intervention with different n3 PUFA sources (with similar proportions of SFA, MUFA and PUFA) on various MetS parameters and systemic inflammation in *fa/fa* Zucker rats by analyzing:

a) Glycemia and insulinema (fasting serum glucose and insulin concentrations)

b) Insulin sensitivity (oral glucose tolerance test [OGTT], area under the curve [AUC] for glucose and insulin, and homeostasis model assessment of insulin resistance [HOMA-IR])

c) Markers of hepatic glucose and insulin uptake (PTP1B and FGF-21 protein levels)

d) Obesity (body weights, weight gain, fat pad mass and fat pad/body weight ratios and body composition)

e) Lipidemia (fasting serum TAG, HDL-C, LDL-C and total cholesterol)

f) Hypertension (tail cuff measurements)

RESEARCH DESIGN AND METHODOLOGY

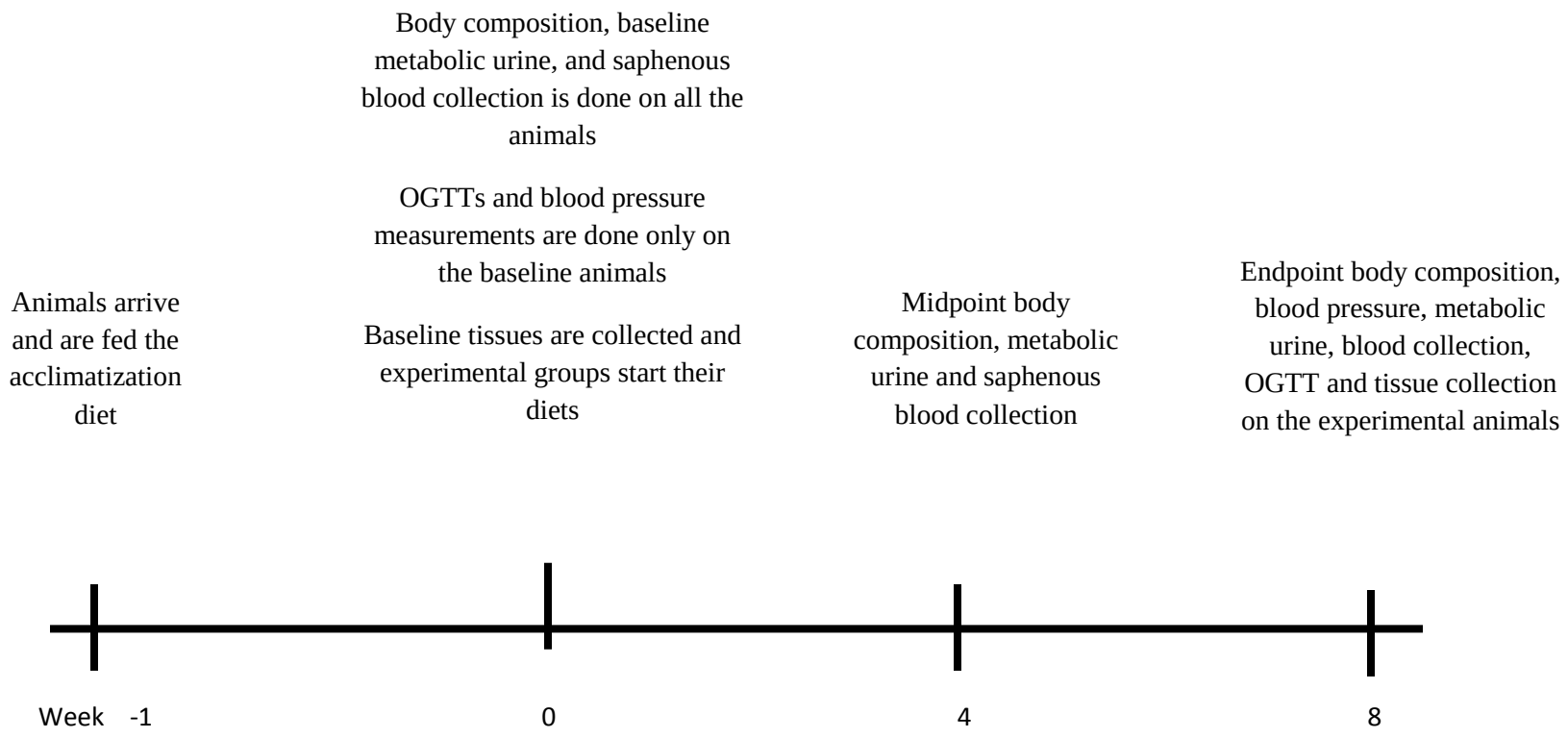
Animals and diets

Five week old male *fa/fa* Zucker and lean Zucker rats were obtained from Charles River Laboratories (St-Constant, PQ). After a minimum 1 week acclimation period, rats were randomly assigned to one of the following groups (n=10 rats/group):

1. Baseline *fa/fa* Zucker fed a diet containing soybean oil (faBASE; based on the AIN-93G diet), where assessments and tissues collections were completed at the end of the acclimation period
2. *fa/fa* Zucker rats fed diet containing n3 PUFA from ALA (faALA; canola/flaxseed oil blend) for 8 weeks
3. *fa/fa* Zucker rats fed diet containing n3 PUFA from EPA (faEPA) for 8 weeks
4. *fa/fa* Zucker rats fed diet containing n3 PUFA from DHA (faDHA) for 8 weeks
5. *fa/fa* Zucker rats fed diet containing n6 PUFA from LA (faSAFF; safflower oil) for 8 weeks
6. Lean Zucker rats fed diet containing n6 PUFA from LA (lnSAFF; safflower oil) for 8 weeks. This group will serve as the healthy reference group.

The diets were based on the AIN-93G diet (Reeves et al., 1993). The fatty acid composition of each oil was determined by gas chromatography and this information used to design oil mixtures that met the goals of varying the n3 PUFA composition while maintaining similar proportions of SFA, MUFA and PUFA in each diet. The diet formulations and the fatty acid composition of the diets are shown in Table 6 and 7, respectively. Diet samples were collected during the experiment and fatty acid

composition verified by gas chromatography. The time line for various assessments is summarized in Figure 2.



Each rat was weighed weekly and fed their respective diets 3 times a week

Figure 2: Summary of the experimental design

The diets were fed *ad libitum*. Feed intake, corrected for spillage, and weekly body weights were recorded for all groups. The 8 week intervention is based on the previous experience with the dietary interventions, including flaxseed oil in *fa/fa* Zucker rats (Baranowski et al., 2012). The sample size of n=10 per group is sufficient based on previous experience assessing functional parameters such as oral glucose tolerance as well as the various biochemical and molecular markers.

Mammals are unable to synthesize LA and ALA, thus they are considered the essential n6 and n3 fatty acids in the diet (Hibbeln et al., 2006). n6 fatty acids are found in most vegetable oils, nuts and grains. Most Western diets have an n6:n3 ratio >10:1, with the average ratio in the Western diet being 15:1 (Hibbeln et al 2006). Soybean oil has an n6:n3 ratio of 6:1. It is the most commonly consumed vegetable oil in the world and it is the most commercially used oil in the food industry. Soybean oil is also the oil that is used in the AIN-93G diets to provide a balanced n6:n3 ratio and to promote growth in experimental rats. Thus, soybean oil was chosen as the fat source for the acclimatization diet.

High linoleic acid safflower was used in the control diet since it is high in n6 and low in n3 as indicated by the high n6:n3 ratio (240:1). The safflower diet was fed to both the lean and obese control groups to allow for a comparison not only among diets for the *fa/fa* groups, but also a comparison in between genotypes since the lean Zucker rats were also fed the safflower diet.

The hypothesis stated above is that n3 from a plant source is as effective as n3 from marine sources for reducing parameters of MetS and hepatic steatosis. A canola/flaxseed oil diet was previously shown to successfully decrease hepatic steatosis in a diet-induced model (DIO) of obesity (Hanke et al., 2013). In this study, the diet that

contained 87% flax oil, 10% canola oil, and 3% coconut oil created an oil mixture that was high in n3 PUFAs but also high in MUFAs. By adding 10% canola oil, the oil mixture had almost 20% MUFA, which makes it useful for food applications such as frying, while still maintaining a 1:3 n6 to n3 ratio. The addition of the canola oil also kept the MUFA amount similar among all the experiment diets. The 3% coconut oil was added to the diet to keep the SFA content of every experimental diet consistent.

There have been various studies that show the beneficial effects of marine oils on obesity and hepatic steatosis (Ahmed & Byrne, 2009). Most of these studies have used marine oil containing a mixture of EPA and DHA; there are limited studies comparing EPA-rich oil versus DHA-rich oil. By using purified forms of EPA or DHA in the diet, we were able to determine which configuration of n3 PUFAs are associated with these beneficial effects. Soybean oil was added to the diet to keep the SFA and MUFA content consistent amongst the diets. The ratio of soy oil:EPA oil was 67:33 and this results in 3% of the overall weight in the diet from EPA as the diets contained 10% (w/w) total fat. A study done by Sankaran et al. (2004) found that there was an increase in complications including renal failure in *pcy* mice fed a diet that was 5% (w/w) DHA. Due to this, we did not use a diet higher than 3% (w/w) DHA, and to allow for comparison with an equal dose of EPA, the EPA diet also had 3% (w/w) EPA. The purified EPA and DHA oils used in this project were in FFA form and contained >95% EPA or DHA, respectively.

To parallel the EPA diet, the diet that contained DHA was a blend of 67:33 soybean oil to DHA. This allowed us to compare diets with similar content of EPA and DHA by formulating diets that were consistent in proportions of SFA, MUFA and PUFA.

It has been suggested that humans have evolved on a diet with a 1:1 ratio of n6:n3 and that is the ideal ratio to prevent chronic diseases (Simopoulos, 2006). Furthermore, in both rats and humans the recommended adequate intakes of PUFA that prevent biochemical and physiological symptoms of deficiency and optimize tissue PUFA content correspond to approximately 2% of energy in the diet for LA and 0.5% of energy in the diet from ALA (Guesnet et al., 2011).

Table 6: Diet formulation

Diet Ingredients (g/kg) [a]	Soybean Oil Diet	Safflower Oil Diet	Canola/ Flaxseed/ Coconut Oil Diet	EPA/ Soybean Oil Diet	DHA/ Soybean Oil Diet
Cornstarch	348	348	348	348	348
Maltodextrin	132	132	132	132	132
Sucrose	100	100	100	100	100
Egg white	213	213	213	213	213
Cellulose	50	50	50	50	50
AIN-93G-MX mineral mix	35	35	35	35	35
AIN-93-VX vitamin mix	10	10	10	10	10
Choline	3	3	3	3	3
Biotin mix[b]	10	10	10	10	10
Soybean oil	100	0	0	67	67
Safflower oil[c]	0	100	0	0	0
Flaxseed oil [d]	0	0	87	0	0
Canola oil [e]	0	0	10	0	0
Coconut oil [f]	0	0	3	0	0
EPA oil [g]	0	0	0	33	0
DHA oil [g]	0	0	0	0	33

[a] Ingredients from Dyets, Inc (Bethleham, PA) unless otherwise indicated
[b] 200 mg biotin/kg cornstarch was added because egg white was the protein source and avidin binds to biotin
[c] Alnor Oil Company (Valley Stream. NY)
[d] Omega Nutrition (Vancouver, BC)
[e] Smuckers Food Services (Markham, ON)
[f] Nutiva (Richmond, CA)
[g] Larodan Fine Chemicals (Malmö, SE)

Table 7: Fatty acid composition of the experimental diets

Fatty Acid Composition (g/100 g)	Soybean Oil	Safflower Oil	Flaxseed/ Canola/ Coconut Oil (87:10:3)	EPA/ Soybean Oil (33:67)	DHA/ Soybean Oil (33:67)
SFA	15	10	11	10	10
MUFA	21	17	19	15	15
PUFA	63	72.3	70	75	75
C18:2n6 (LA)	54	72	18	36	36
C18:3n3 (ALA)	9	0.3	52	6	6
C22:5n3 (EPA)	0	0	0	32	0
C22:6n3 (DHA)	0	0	0	0	33
Other n3 PUFAS	0	0	0	0	0
Other n6 PUFAS	0	0	0	0	0
Other PUFAS	0	0	0	1	0
n6:n3	6:1	240:1	1:3	1:1.1	1:1.1

Feed intake

Feed intake was recorded by weighing the feed in and feed out 3 times a week and calculating the difference and correcting for spillage. Weekly feed intake was determined by the sum of the 3 weekly measurements and the total feed intake for the study was determined by the sum of the weekly totals.

Body weight

Body weights were recorded once per week by placing each animal in a polycarbonate container on a scale. Results were graphed to show changes in weights between each dietary group over the course of the study.

Body composition

Body composition of the rats was assessed *in vivo* using an EchoMRI-700™ whole body Quantitative Magnetic Resonance (QMR) instrument (Echo Medical Systems, Houston TX). The rats were placed in restraining tubes appropriate to their size; each measurement took approximately 2 minutes. Measures of body composition (body fat, lean lass, total water and total free water) were obtained for the baseline group, and for each rat starting a diet, at week 0, week 4 and week 8 to allow for progressive comparisons.

Blood pressure

During the baseline week, blood pressure was assessed on the baseline animals using the tail cuff method and CODA system (Kent Scientific, Torrington, CT). The rats

were restrained in a rodent holder containing a darkened nose cone to limit their view and to reduce the level of stress. A spacer was present at the rear of the holder to allow the rat's tail to be free. The procedure room was kept warm (approximately 26^{o}C) and warming devices including a heated platform and a heat lamp were used to maintain the animal's surface body temperature at 30^{o}C. An occlusion cuff was then placed close to the base of the tail followed by a volume pressure recording (VPR) sensor. Five acclimatization blood pressure readings (9 cycles) were taken to help get the rat accustomed to the procedure. Then 15 readings (15 cycles) were taken and used to calculate an average blood pressure reading. All of the equipment is controlled with a computer system. Groups on experimental diets had their blood pressure taken at week 0 and week 8 of the dietary intervention.

Urine and saphenous blood collection

The *fa/fa* Zucker rats develop impaired glucose tolerance as early as 5 weeks of age and this is a risk factor for the development of diabetes (Zucker and Antoniades, 1972). One of the consequences of diabetes is a decrease in kidney function, along with progressive nephropathy over time, which leads to renal damage and end-stage renal failure (reviewed by Lim, 2014). Thus, urinary levels of glucose, creatinine and urea were used as indicators of pre-diabetes progression and renal function.

During the baseline week (baseline group) or during weeks 0 and 8 (intervention groups) the rats were placed in metabolic cages without feed for 5 hours for urine collection. Water was available *ad libium* for the duration of the fast. During week 8, the 5 hour fast was prior to the first time interval for the OGTT. The urine was collected into

pre-weighed vials and urine weight (obtained by difference) was used to determine urine volume during the 5 h period. Urine was analyzed using the cobas c 111 clinical chemistry analyzer (Roche Diagnostics, Indianapolis, IN, USA) for glucose (Roche Diagnostics, Indianapolis, IN, cat # 04657527 190), urinary creatinine (Roche Diagnostics, Indianapolis, IN, cat # 05401755 190) and urea (Roche Diagnostics, Indianapolis, IN, cat # 04657616 190).

On day 0 and week 8 of the study (for the experimental animals), the rats were exposed to a 5 hour fast (in metabolic cages) and fasting blood samples were obtained via the saphenous vein. Blood was collected by shaving the hind limb of each animal and restraining each animal within a towel. The skin was pricked using a sterile 22 gauge needle and blood was collected using microvette tubes (Sarstedt, Montreal, QC). Approximately 200 µl of blood were collected and stored on ice until centrifuged at 1000 g for 15 minutes at 4°C. The serum layer from the saphenous blood collections was then aliquoted and stored at -80°C to be later analyzed.

OGTT and calcuations

Oral glucose tolerance testing was completed on the baseline group during baseline week and on the experimental groups during week 8. The rats had body composition and blood pressure completed the week before their OGTT to minimize changes in blood glucose due to stress. On the day of the test, rats were fasted for 5 hours and an initial blood sample was collected from the saphenous vein (t=0). An oral glucose load (1 g glucose/kg body weight, provided as 70% dextrose solution at 0.00143 mL/g body weight) was administered orally using a plastic syringe (without a needle), followed

by additional blood collection at t=15, t=30, t=60 and t=120 minutes post glucose consumption. The blood samples were stored on ice until centrifuged at 1500 g for 15 minutes at 4°C. The serum layer was aliquoted and stored at -80°C for analysis of glucose and insulin (procedures to be later described).

AUC was calculated using the trapezoidal method (Purves, 1992). The base calculation is as follows, where C = blood glucose concentration:

$$AUC_{time\,1-0} = [(C_0 + C_{1)} \div 2] \times Time_{1-0}$$

AUC was repeated for each time interval and then the sum of all the time intervals was taken. The glucose-insulin index was also calculated using the AUC for both glucose and insulin (Myllynen et al., 1987). The formula is as follows:

$$Glucose - Insulin\ Index = AUC_{glucose} \times AUC_{insulin}$$

HOMA-IR uses fasting glucose and insulin concentrations to evaluate insulin sensitivity (Matthews et al., 1985). HOMA-IR is calculated as follows:

$$HOMA - IR = \frac{Fasting\ serum\ insulin\ \left(\frac{\mu U}{mL}\right) \times\ Fasting\ serum\ glucose\ \left(\frac{mmol}{L}\right)}{22.5}$$

Insulin values were converted from pmol/L to μU/mL using an insulin unit conversion factor of 6.945.

Tissue collection

At the end of the baseline week for the baseline group and at the end of week 8 of the dietary intervention for the experimental groups, the rats were fasted overnight, and euthanized by carbon dioxide asphyxiation, followed by decapitation. Carbon dioxide

asphyxiation was used for this protocol as pentobarbital or isofluorane (and related compounds) may affect liver, glucose and lipid metabolism, and thus confound the interpretation of these parameters and the effects of the dietary intervention. Trunk blood was collected after decapitation and centrifuged at 1000 g for 15 minutes at 4^{o}C. The serum layer was collected and stored at -80°C for future analysis. Tissues such as the liver, pancreas, kidneys, epididymal fat, perirenal fat, and mesenteric fat were weighed and stored accordingly for analysis. Tissues were immediately frozen in liquid nitrogen and stored at -80°C for Western blotting and fatty acid analyses. A small portion of the liver and epididymal adipose tissue were placed into embedding media containing optimal cutting temperature (OCT) compound (Sakura Finetek, Torrance, CA, cat # 25608-930) and cryogenic gel (Electrol Microscopy Sciences, Hatfield, PA, cat # 62806-01), respectively. Both tissue samples were frozen in a dry ice-ethanol bath, and stored at -80°C until used for preparing frozen sections. A small portion of pancreas was fixed in phosphate-buffered formalin for 24 hours, then placed in phosphate-buffered saline and stored at 4°C for preparation of paraffin-embedded sections.

Serum biochemistry

Glucose

One of the risk factors for developing MetS is having an elevated fasting blood glucose level (5.6 mmol/L or higher), with DM2 being diagnosed when fasting blood glucose concentrations are greater than 7.0 mmol/L (reviewed by Salazar et al., 2013). Fasting blood glucose was measured in samples from the saphenous blood collection using a colormetric assay (Genzyme Diagnostics Glucose Reagent and Glucose

Calibrator, cat #220-32, Charlottetown, PE, Canada; SEKISUI Diagnostics DC-CAL Calibrator, cat # SE-035, North Vancouver, B.C., Canada) as only small quantities of blood were collected from the saphenous vein.

Test Principle

The procedure allows for the measurement of glucose using the glucose oxidase/peroxidase (GOD-POD) method as follows:

$$\text{B-D-glucose} + O_2 + H_2O \xrightarrow[\text{Glucose Oxidase}]{} \text{D-gluconic Acid} + H_2O_2$$

$$H_2O_2 + \text{hydroxybenzoate} + \text{4-aminoantipyrine} \xrightarrow[\text{Peroxidase}]{} \text{Quinoeimine Dye} + H_2O$$

The amount of red dye produced is measured at 505 nm and is proportional to the amount of glucose present in the sample.

Reagents

1. Quality control solution was prepared according to instructions: 3 mL of DC-CAL Diluent was added to DC-CAL Calibrator, swirled gently and allowed to sit for 30 minutes. DC-CAL quality control solution was mixed by inverting before use.
2. Glucose reagent was prepared according to the instructions: 100 mL of double distilled water (ddH_2O) was added to the dry reagent, swirled gently and allowed to sit for 5 minutes.
3. All solutions and samples were thawed and kept on ice (for all assays to follow).
4. The glucose stock calibration solution (5 mmol/L) was used to prepare the standard curve by adding 50 µL of stock to 50 µL of ddH_2O to give a 2.5 mmol/L

working solution. This serial dilution was repeated until all concentrations for the standard curve were reached: 0.625, 1.25, 2.5, 5 mmol/L with ddH_2O used as a blank (0).

Procedure

Samples and the quality control were diluted 5 times (ie: mixing 7 µL sample and 28 µL ddH_2O). All samples, quality control, blank and calibration solutions were vortexed before pipetting 5 µL into a NUNC 96 well plate (NUNC^{TM}, Roskilde, Denmark, cat # 167008) in triplicate. Next, 200 µL of glucose reagent was added to each well using a multichannel pipette. The plate was then swirled gently to mix the solution and allowed to incubate at room temperature for 10 minutes. After incubation, the plate was read on the FLUOstar Omega (BMG LABTECH, Ortenberg, Germany) plate reader at an absorbency of 505 nm. Any samples with a CV greater than 10% were not used and the assay was repeated.

Insulin

Insulin resistance refers to a state where cells are less responsive to insulin. To maintain the normal biological actions of insulin thus requires greater production of insulin by the β-cells in the pancreas, which can lead to hyperinsulinemia (reviewed by Machado and Cortez-Pinto, 2014). Furthermore, as the body becomes resistant to insulin, glucose levels in the circulation rise, thus leading to hyperglycemia. Insulin resistance is strongly associated with obesity and hepatic steatosis and it is seen as an important risk factor for NAFLD.

Test principle

Meso-Scale Discovery (MSD) coats an antibody for protein of interest onto one electrode or spot in the well. Samples are added to each well and the insulin in the sample binds to the antibody on the electrode surface on the bottom of each well. An electochemoluminsecent labelled antibody is then added and binds to the insulin that was captured by the antibody on the plate, creating a sandwich immunoassay. A read buffer is then added to provide the environment for the electrochemiluminesence. When loaded into the MSD instrument, a voltage is applied to the plate electrodes causing the labelled antibody to emit light. The intensity of the light emitted corresponds to the amount of insulin in the sample.

Reagents

1. MSD Mouse/Rat Insulin Kit (cat. # K152BZC-2, Gaithersburg, MD).
2. Prepared Blocker A solution according to instructions using ultra pure water (cat. #400000, lot. # 0430425-1, Cayman Medical Company, Ann Arbor, MI).
3. Prepared 1 L of wash buffer (0.05% Tween-phosphate buffered saline [PBS-T]) by adding 0.5 mL of Tween 20 (cat. #P5927-500mL, Sigma Aldrich, St. Louis, MO) to 1 L of PBS (cat. #P5368, lot# 081M82223, Sigma Aldrich, St. Louis, MO).
4. Diluted the insulin calibrator to create the first standard by adding 10 µL of the stock solution to 990 µL of diluent 100 and vortexing to mix. The remaining 6 standards were prepared by a 3-fold serial dilution where 100 µL of the previous

standard was added to 200 µL to diluent 100. The last standard was a zero calibrator (diluent 100).

5. Prepared detection antibody by adding 50 µL of the 100× detection antibody solution to 4.95 mL of diluent 17 in a 15 mL conical tube, to create a 1× detection antibody solution.
6. Prepared read buffer by adding 5 mL of 4× read buffer to 15 mL of ultra pure water in a 50 mL conical tube.

Procedure

1. 150 µL of Blocker A was added to each well on the MSD plate using a multichannel pipette. The plate was then sealed with an adhesive plate seal and incubated for 1 hour at room temperature with vigorous shaking (300-1000 rpm).
2. The plate was washed 3 times with 0.05% PSB-T and then the plate was tapped onto paper towel after the last wash to ensure all liquid was expelled from the wells.
3. 40 µL of the detection antibody was added to each well using a multichannel pipette and reverse pipetting. Reverse pipetting is a technique used to ensure there was no bubble formation. The solution was aspirated into the pipette tip so that there is a greater volume of the sample in the tip than would be delivered to the plate. This ensures that liquid that will remain in the tip after expelling the desired amount of liquid from the tip, preventing bubble formation.
4. 10 µL of calibrator or sample (*fa/fa* samples diluted 1:5 with ultra pure water and lean samples were undiluted) was added to the wells in duplicate. The plate was sealed with an adhesive plate seal, covered in aluminum foil to prevent

penetration from light, and incubated at room temperature for 2 hours with vigorous shaking (300-1000 rpm).

5. During the last incubation a system test plate was run on the MSD Sector ® Imager 2400 (Meso-Scale Discovery, Rockville, MD) to make sure it was working correctly.
6. The plate was washed 3 times with 0.05% PBS-T.
7. 150 µL of 1× read buffer was added to each well by reverse pipetting. The plate was immediately read on the Sector ® Imager 2400 using MSD Discovery Workbench ® analysis software.

The standard curve range was from 0-50000 pg/mL. Only standards and samples with a CV of less than 20% were used. The lower limit of detection (LLOD) was the calculated concentration of the signal that was 2.5 standard deviations over the zero calibrator. Percent recovery was calculated as the measured concentration divided by the previous dilution (expected):

$$\%\, Recovery\ = \frac{measured\ \times\ dilution\ factor}{expected\ \times 100}$$

Percent recovery for the standards was between 80-120 or the plate was redone.

Lipid profile

Dyslipidemia is one of the factors related to MetS and hepatic steatosis (reviewed by Machado and Cortez-Pinto, 2014). Total cholesterol (TC, Cholesterol generation 2, Roche Diagnostics, cat. # 04718917 190), HDL-cholesterol (HDL-C, plus 3rd generation, Roche Diagnostics, Indianapolis, IN, cat. # 0541488 190), LDL-cholesterol cholesterol

(LDL-C, plus 2nd generation, Roche Diagnostics, Indianapolis, IN, cat. # 05401682 190), and triglycerides (TAG, Roche Diagnostics, Indianapolis, IN, cat. # 04657597 190) were measured using the cobas c 111 clinical chemistry analyzer.

Haptoglobin

Haptoglobin is the predominant circulating acute phase protein in rats and is an assessment for chronic low grade inflammation, which is associated with MetS (reviewed by Badawi et al., 2010). Trunk serum was analyzed for haptoglobin using an enzymatic colormetric assay kit from Tridelta Development Ltd. (Maynooth Ireland, cat #TP801).

Test Principle

Free hemoglobin exhibits peroxidase activity, which is inhibited at low pH. Haptoglobin present in the sample combines with hemoglobin and at a low pH preserves the peroxidase activity of the bound hemoglobin. Preservation of the peroxidase activity of hemoglobin is directly proportional to the amount of haptoglobin present in the sample.

Reagents

1. Reagent 1: Hemoglobin
2. Reagent 2: Chromogen
3. Sample/Calibrator Diluent: Phosphate-buffered saline diluent
4. Calibrator: A stock solution containing 2.5 mg/mL haptoglobin. The calibrator solution was serially diluted to produce 3 additional standards at concentrations of 1.25, 0.625, 0.312 mg/mL and a blank (0 mg/mL).

Procedure

1. Standards and samples (undiluted) were vortexed and 7.5 µL of each was pipetted in duplicate into a 96-well plate (NUNC ™, Roskide Denmark, cat # 167008).
2. Using a multichannel pipette, 100 µL of Reagent 1 was added to each well and samples were mixed by pipetting up and down 3 times.
3. Using a multichannel pipette, 140 µL of Reagent 2 was added to each well and samples were again mixed by pipetting up and down 3 times.
4. Samples were then incubated for 5 minutes at room temperature, during which all bubbles were popped using a pipette tip.
5. After incubation, the absorbency of each well was immediately measured at 630 nm on a FLUOstar omega plate reader (BMG LABTECH, Ortenberg, Germany).

Note: The normal range for rats is 0.25-0.51 mg/mL, and the acute range for infection is 0.8-1.8 mg/mL.

Monocyte chemattractant protein (MCP)-1

MCP-1 is a cytokine that signals the infiltration of monocytes and T-cells to the site of tissue inflammation. Increased circulating levels of MCP-1 have been shown to be present in obesity, DM2 and insulin resistance (Desmane et al., 2014).

Test principle

Refer to Insulin.

Reagents

1. MSD Rat MCP-1 Cytokine Assay Ultra Sensitive Kit (cat. # K153AYC-1, Gaithersburg, MD).
2. Prepared 1 L of wash buffer (0.05% Tween-phosphate buffered saline [PSB-T]) by adding 0.5 mL of Tween 20 (cat. #P5927-500mL, Sigma Aldrich, St. Louis, MO) to 1 L of PBS (cat. #P5368, lot# 081M82223, Sigma Aldrich, St. Louis, MO).
3. Prepared the calibration curve by transfering 10 µL of the MCP-1 calibrator to 240 µL of diluent 6 and vortexing to mix. Further dilutions were created by transferring 50 µL of the new dilution to 150 µL of diluent 6. This was repeated 5 additional times to generate 7 standards. The blank was diluent 6.
4. Prepared detection antibody by adding 60 µL of the stock antibody solution to 2.95 mL of diluent 5.
5. Prepared read buffer by adding 10 mL of 4× read buffer T to 10 mL of ddH_2O.

Procedure

1. 25 µL of diluent 6 was pipetted with a multichannel pipette via reverse pipetting into each well. The plate was then sealed and left to incubate with vigorous shaking (300-1000 rpm) at room temperature for 30 minutes.
2. 25 µL of undiluted sample or calibrator was added in duplicate, and the plate was sealed and allowed to incubate for two hours with vigorous shaking at room temperature.

3. The plate was washed 3 times with 0.05% PSB-T and then the plate was tapped onto paper towel after the last wash to ensure all liquid was expelled from the wells.
4. 25 µL of 1× detection antibody solution was dispensed into each well through a multichannel pipette using the reserve pipetting technique and the plate was then sealed and allowed to incubate with vigorous shaking for another 2 hours.
5. During the last incubation a system test plate was run on the MSD Sector ® Imager 2400 (Meso-Scale Discovery, Rockville, MD) to make sure it was working correctly.
6. The plate was washed 3 times with 0.05% PBS-T.
7. 150 µL of 2× read buffer was added to each well via reverse pipetting and the plate was immediately read on the Sector ® Imager 2400 using MSD Discovery Workbench ® analysis software.

The standard curve range was from 0-40000 pg/mL.

Refer to Insulin for calculation procedures.

Percent recovery for the standards was between 80-120 or the plate was redone.

Adiponectin

Adiponectin is a protein that is exclusively secreted by mature adipocytes and plasma adiponectin concentrations show a negative correlation with body fat mass (Kratz et al., 2008; Stryjecki and Mutch, 2011). Furthermore, previous studies have shown that an increase in circulating adiponectin correlates with a decrease in circulating insulin (Flasch et al., 2006).

Test Principle

Refer to Insulin.

Reagents

1. Meso-Scale Discovery (MSD) Rat Adiponectin Kit (cat. # K152BXC-1, Gaithersburg, MD)
2. Prepared Blocker A solution according to instructions using ultra pure water (cat. #400000, lot. # 0430425-1, Cayman Medical Company, Ann Arbor, MI).
3. Prepared 1 L of wash buffer (0.05% Tween-phosphate buffered saline [PSB-T]) by adding 0.5 mL of Tween 20 (cat. #P5927-500mL, Sigma Aldrich, St. Louis, MO) to 1 L of PBS (cat. #P5368, lot# 081M82223, Sigma Aldrich, St. Louis, MO)
4. Prepared adiponectin calibrator by adding 10 µL of the stock solution to 490 µL of diluent 100. The next calibrators were prepared by transferring 100 µL of the diluted calibrator to 200 µL of diluent 100. The 3-fold serial dilution was repeated 5 additional times to generate 7 calibrators, with the blank being diluent 100.
5. Prepared detection antibody by adding 30 µL of the 100× detection antibody solution to 2.97 mL of diluent 100 in a 15 mL conical tube, to create a 1× detection antibody solution.
6. Prepared read buffer by adding 5 mL of 4× read buffer to 15 mL of ultra pure water in a 50 mL conical tube.

Procedure

1. 150 µL of Blocker A was added to each well on the MSD plate using a multichannel pipette.
2. The plate was then sealed with an adhesive plate seal and incubated for 1 hour at room temperature with vigorous shaking (300-1000 rpm).
3. The plate was washed 3 times with 0.05% PSB-T and then the plate was tapped onto paper towel after the last wash to ensure all liquid was expelled from the wells.
4. 40 µL of diluent 100 was added to each well using a multichannel pipette and reverse pipetting.
5. 10 µL of calibrator or sample was added to the wells in duplicate. The sample dilution was prepared by two serial dilutions. The first dilution required 10 µL of the sample to be diluted in 90 µL of diluent 100. The second dilution required 10 µL of the first dilution to be added to 990 µL of diluent 100. This dilution was performed for both *fa/fa* animals and lean animals.
6. The plate was sealed with an adhesive plate seal, covered in aluminum foil to prevent penetration from light, and incubated at room temperature for 2 hours with vigorous shaking (300-1000 rpm).
7. The plate was washed 3 times with 0.05% PBS-T.
8. 25 µL of 1× detection antibody solution was added to each well by reverse pipetting and incubated with vigorous shaking (300-1000 rpm) for 2 hours at room temperature.

9. During the last incubation a system test plate was run on the MSD Sector ® Imager 2400 (Meso-Scale Discovery, Rockville, MD) to make sure it was working correctly.
10. The plate was washed 3 times with 0.05% PBS-T.
11. 150 µL of 1X read buffer was added to each well via reverse pipetting and the plate was immediately read on the Sector ® Imager 2400 using MSD Discovery Workbench ® analysis software.

The standard curve range was from 0-200 ng/mL.

Refer to Insulin for calculation procedures

Percent recovery for the standards was between 80-120 or the plate was redone.

Leptin

Leptin is a product of the *ob* gene and is produced and released by adipocytes. It plays a key role in metabolism and regulation of adipose tissue (reviewed by Matarese et al., 2005). Leptin is released in amounts that mirror overall body fat stores and acts on neurons and hypothalamic receptors, thereby influencing the brain's perception of nutritional energy status and appetite (Coll et al, 2007). The absence of functional leptin or its receptors leads to uncontrolled food intake and results in obesity, as seen in the *fa/fa* Zucker rats.

Test principle

Refer to Insulin.

Reagents

1. MSD Rat Leptin Kit (cat. # K153BYC-1, Gaithersburg, MD).
2. Prepared Blocker A solution according to instructions using ultra pure water (cat. #400000, lot. # 0430425-1, Cayman Medical Company, Ann Arbor, MI).
3. Prepared 1 L of wash buffer (0.05% Tween-phosphate buffered saline [PSB-T]) by adding 0.5 mL of Tween 20 (cat. #P5927-500mL, Sigma Aldrich, St. Louis, MO) to 1 L of PBS (cat. #P5368, lot# 081M82223, Sigma Aldrich, St. Louis, MO).
4. Prepared the leptin calibrator by adding 10 µL of the stock solution to 990 µL of diluent 6. Prepared the next calibrator by transferring 100 µL of the diluted calibrator to 200 µL of diluents 6. Repeated 3-fold serial dilution 5 additional times to generate 7 calibrators, with the blank being only diluent 6
5. Prepared detection antibody by adding 30 µL of the 100× detection antibody solution to 2.97 mL of diluent 100 in a 15 mL conical tube, to create a 1× detection antibody solution.
6. Prepared the read buffer by adding 5 mL of 4× read buffer to 15 mL of ultra pure water in a 50 mL conical tube.

Procedure

1. 150 µL of Blocker A was added to each well on the MSD plate using a multichannel pipette.
2. The plate was then sealed with an adhesive plate seal and incubated for 1 hour at room temperature with vigorous shaking (300-1000 rpm).

3. The plate was washed 3 times with 0.05% PSB-T and then the plate was tapped onto paper towel after the last wash to ensure all liquid was expelled from the wells.
4. 40 µL of diluent 6 was added to each well using a multichannel pipette and reverse pipetting.
5. 10 µL of calibrator or undiluted sample was added to the well in duplicate.
6. The plate was sealed with an adhesive plate seal, covered in aluminum foil to prevent penetration from light, and incubated at room temperature for 2 hours with vigorous shaking (300-1000 rpm).
7. The plate was washed 3 times with 0.05% PBS-T.
8. 25 µL of 1× detection antibody solution was added to each well by reverse pipetting and incubated with vigorous shaking (300-1000 rpm) for 1 hour at room temperature.
9. During the last incubation a system test plate was run on the MSD Sector ® Imager 2400 (Meso-Scale Discovery, Rockville, MD) to make sure it was working correctly.
10. The plate was washed 3 times with 0.05% PBS-T.
11. 150 µL of 1× read buffer was added to each well via reverse pipetting and the plate was immediately read on the Sector ® Imager 2400 using MSD Discovery Workbench ® analysis software.

The standard curve range was from 0-100000 pg/mL.

Refer to Insulin for calculation procedures.

Percent recovery for the standards was between 80-120 or the plate was redone.

Resistin

Resistin is a 12.5 kDa peptide hormone, synthesized by adipocytes and immune cells whose physiological properties are linked to obesity and DM2 (reviewed by McTeran et al., 2006). Rajala et al. (2003) found that the administration of resistin *in vivo* and transgenic overexpression of resistin induces insulin resistance by increasing hepatic glucose production in Sprague Dawley rats.

Test Principle

Refer to Insulin.

Reagents

1. MSD Mouse/Rat Resistin Kit (cat #L152FNC-1)
2. Prepared Blocker A solution according to instructions using ultra pure water (cat. #400000, lot. # 0430425-1, Cayman Medical Company, Ann Arbor, MI).
3. Prepared 1 L of wash buffer (0.05% Tween-phosphate buffered saline [PSB-T]) by adding 0.5 mL of Tween 20 (cat. #P5927-500mL, Sigma Aldrich, St. Louis, MO) to 1 L of PBS (cat. #P5368, lot# 081M82223, Sigma Aldrich, St. Louis, MO).
4. Prepared resistin calibrator to a concentration of 0.1 µL by adding 10 µL of the stock solution to 990 µL of diluent 11. Prepared the highest concentrations of the resistin calibrator for the standard curve by adding 50 µL of the diluted resistin calibrator previously prepared to 350 µL of diluent 11 and vortexed to mix.

Prepared the next calibrator by transferring 100 µL of the diluted calibrator to 200 µL of diluent 11. Repeated 3-fold serial dilution 5 additional times to generate 7 calibrators, with the 8th standard (blank) being only diluent 11.

5. Prepared detection antibody by adding 30 µL of the 100× detection antibody solution to 2.97 mL of diluent 11 in a 15 mL conical tube, to create a 1× detection antibody solution.
6. Prepared read buffer by adding 5 mL of 4× read buffer to 15 mL of ultra pure water in a 50 mL conical tube.

Procedure

1. 150 µL of Blocker A was added to each well on the MSD plate using a multichannel pipette.
2. The plate was then sealed with an adhesive plate seal and incubated for 1 hour at room temperature with vigorous shaking (300-1000 rpm).
3. The plate was washed 3 times with 0.05% PSB-T and then the plate was tapped onto paper towel after the last wash to ensure all liquid was expelled from the wells.
4. 40 µL of diluent 18 was added to each well using a multichannel pipette and reverse pipetting.
5. 10 µL of calibrator or sample (all samples were diluted 1:5 with diluent 11) were added to the wells in duplicate. The plate was sealed with an adhesive plate seal, covered in aluminum foil to prevent penetration from light, and incubated at room temperature for 2 hours with vigorous shaking (300-1000 rpm).

6. The plate was washed 3 times with 0.05% PBS-T.
7. 25 µL of 1× detection antibody solution was added to each well by reverse pipetting and incubated with vigorous shaking (300-1000 rpm) for 2 hours.
8. During the last incubation a system test plate was run on the MSD Sector ® Imager 2400 (Meso-Scale Discovery, Rockville, MD) to make sure it was working correctly.
9. The plate was washed 3 times with 0.05% PBS-T.
10. 150 µL of 1× read buffer was added to each well via reverse pipetting and the plate was immediately read on the Sector ® Imager 2400 using MSD Discovery Workbench ® analysis software.

The standard curve range was from 0-12500 pg/mL.

Refer to Insulin for calculation procedures.

Percent recovery for the standards was between 80-120 or the plate was redone.

TNF-α

As a result of obesity, there is an increase in adipocyte size and adipose cell dysfunction which consequently leads to an imbalance of adipose derived pro-inflammatory markers, including TNF-α (reviewed by Jung and Choi, 2014).

Test principle

Refer to Insulin.

Reagents

1. MSD Rat TNF- α Cytokine Assay Ultra Sensitive Kit (cat. # K153BHC-1, Gaithersburg, MD)
2. Prepared 1 L of wash buffer (0.05% Tween-phosphate buffered saline [PBS-T]) by adding 0.5 mL of Tween 20 (cat. #P5927-500mL, Sigma Aldrich, St. Louis, MO) to 1 L of PBS (cat. #P5368, lot# 081M82223, Sigma Aldrich, St. Louis, MO).
3. Prepared calibration curve by transferring 10 µL of the TNF-α calibrator to 240 µL of diluent 6 with vortexing to mix. Further dilutions were created by transferring 50 µL of the new dilution to 150 µL of diluent 6. This was repeated 5 additional times to generate 7 standards. The blank was diluent 6.
4. Prepared detection antibody by diluting 60 µL of the stock antibody solution with 2.95 mL of diluent 5.
5. Prepared read buffer by adding 10 mL of 4× read buffer T to 10 mL of ddH_2O.

Procedure

1. 25 µL of diluent 6 was pipetted with a multichannel pipette via reverse pipetting into each well. The plate was then sealed and left to incubate with vigorous shaking (300-1000 rpm) at room temperature for 30 minutes.
2. 25 µL of undiluted sample or calibrator was added in duplicate, and the plate was sealed and allowed to incubate for two hours with vigorous shaking at room temperature.

3. The plate was washed 3 times with 0.05% PSB-T and then the plate was tapped onto paper towel after the last wash to ensure all liquid was expelled from the wells.
4. 25 µL of 1× detection antibody solution was dispensed into each well through a multichannel pipette using the reserve pipetting technique and the plate was then sealed and allowed to incubate with vigorous shaking for another 2 hours.
5. During the last incubation, a system test plate was run on the MSD Sector ® Imager 2400 (Meso-Scale Discovery, Rockville, MD) to make sure it is working correctly.
6. The plate was washed 3 times with 0.05% PBS-T.
7. 150 µL of 2× read buffer was added to each well via reverse pipetting and immediately read on the Sector ® Imager 2400 using MSD Discovery Workbench ® analysis software.

The standard curve range was from 0-40000 pg/mL.

Refer to Insulin for calculation procedures.

Percent recovery for the standards was between 80-120 or the plate was redone.

ALT and AST

AST and ALT are enzymes present in the liver. An increased level of circulating AST and ALT indicates that hepatic function has been compromised leading to the disruption of hepatocytes and allowing AST and ALT to leak into the bloodstream (reviewed by Scheig, 1996). AST (Roche Diagnostics, Indianapolis, IN, cat # 04657543 190) and ALT (Roche Diagnostics, Indianapolis, IN, cat # 04718569 190) were analyzed

using the cobas c 111 clinical chemistry analyzer (Roche Diagnostics, Indianapolis, IN, USA).

Hepatic total lipid concentration

Hepatic steatosis, which is the accumulation of intrahepatic fat, is the least severe form of NAFLD (reviewed by Garg and Misra et al., 2002). Liver total lipid was determined by a modified Folch method, which uses solvent extraction to separate the lipids from the rest of the tissue constituents (Folch et al., 1954).

One and a half grams of liver was weighed and homogenized in 32 mL of 2:1 chloroform:methanol with 0.01% butylated hydroxytoluene (BHT) for 60 seconds with a PT-MR Polytron 1600E. Twenty-two mL of the homogenate was filtered through a #1 Whatman filter paper into a 25 mL graduated cylinder for total hepatic lipid analysis and 6 mL of the homogenate was filtered through a #1 Whatman filter paper into a 15 mL test tube for fatty acid analysis. Afterwards, 0.73% sodium choloride (NaCl) was added at a volume of 10% of the volume of the homogenate in the graduated cylinder designated for hepatic total lipid analysis. The cylinder was stoppered and the mixture inverted 5 times and left to separate overnight. Additionally, 20 mL glass scintillation vials were placed in the desiccator for use the following day.

The next day, the volume of the lower chloroform/lipid layer was recorded and 10 mL of this layer was dried in preweighed 20 mL glass vials. The chloroform was evaporated under nitrogen at 30°C with an N-EVAP III Nitrogen evaporator. The 20 mL glass vials that contained the lipid were dried in the desiccator overnight.

The next day the vials were weighed and the lipid concentrations were calculated as follows:

$$\% \, lipid = \frac{[(dry\ weight\ of\ vial + lipid) - (dry\ weight\ vial)]}{10\ mL\ chloroform\ used} \times volume\ of\ chloroform\ layer \times 100$$

Hepatic fatty acid composition

Hepatic fatty acid composition was determined using a series of procedures. The lipids were first extracted using a modified Bligh and Dyer extraction procedure (Bight & Dyer, 1956). From there, the lipid samples, suspended in 2:1 chloroform methanol, were spotted on a Whatman K8 Silica Gel 80A plate to separate the PL fraction from the TAG fraction. The separate fractions were methlyated in preparation for analysis of fatty acid composition via gas chromatography. Through this we are able to see the differences in hepatic fatty acid composition as they are related to the various experimental diets. All the solvents used in fatty acid analysis were high performance liquid chromatography (HPLC) grade from Fisher Scientific to ensure maximum purity and minimize contamination.

Extraction

To the 6 mL of filtered homogenate (from hepatic total lipid concentration), 2.3 mL of 0.73% NaCl was added. The mixture was vortexed for 30 seconds and then centrifuged with a Sorvall ST 16R centrifuge for 10 minutes at 1000 g. Once completed, the top layer, which consisted of methanol and water, was removed from each sample and

discarded. The remaining bottom layer was rinsed twice with 1-2 mL of a theoretical upper phase of partition (TUP) (3:48:47 chloroform:methanol:water) with the top layer being removed and discarded each time. The sample was then transferred into a clean 8 mL test tube and evaporated under nitrogen to dryness in a 30^{o}C water bath. When the samples were dry they were diluted with 2 mL of 2:1 chloroform: methanol with 0.01% BHT. The PL and TAG were isolated by thin layer chromatography.

Thin layer chromatography (TLC)

A Whatman K8 Silica Gel 80A plate was scored to create lanes and prevent the samples from merging. The plate was activated by heating in a preheated 120^{o}C oven for 30 minutes and cooled in a desiccator for 30 minutes. Once cool the plate was spotted with the appropriate lipid sample containing the PL and TAG standards.

Two hundred µL of lipid extract was combined with both 10 µl of PL internal standard (1.4 mg/mL 1.2-dipetadecanoyl-sn-glycero-3 phosphatidylcholine [Avanti Polar Lipids, Alabaster, AL, cat #850350P]) and 5 µL of TAG internal standard (5 mg/mL triheptadecanoin [Nu-Chek Prep. Inc., Waterville, MN, cat # T-155]). The lipid extract containing both standards, was dried at 30°C with a nitrogen evaporator, reconstituted with 50 µL 2:1 chloroform: methanol and then spotted onto a TLC plate in 4 cm bands. Before and between each sample the application needle was rinsed 3 times with 2:1 chloroform:methanol to avoid cross contamination. Once the plate was spotted it was placed into a pre-prepared chromatography tank containing 80:20:1 petroleum ether:ethyl ether:acetic acid (v/v/v) and then closed. The plate was in the chamber for 25-35 minutes, or until the solvent advanced to about half an inch from the top of the plate. The plate

was then removed from the tank to allow it to air dry, and then it was sprayed with 0.1% 8-anilin-1 napthalene sulfonic acid. The bands for the different lipid classes were illuminated under UV light and marked lightly with a pencil. With the use of a razor blade, the PL and the TAG bands were scraped from the plate onto weigh paper and transferred to a clean 8 mL test tube containing 1 mL toluene with 0.1% BHT. The test tubes were then flushed with nitrogen and capped and stored at -20°C, or the samples proceeded directly to the methylation step.

Methylation

A total of 1.2 mL of 0.3 M methanolic hydrocholoric acid (HCl) (Supelco, Bellefonte, PA cat # 33050-U) was added to each sample. The test tubes were then capped tightly and vortexed for 30 seconds and then placed in an oven preheated to 80°C for an hour. After the tubes had cooled, 1 mL of deionized water was added to each sample. The tubes were then capped and vortexed for 30 seconds and then centrifuged for 10 minutes at 1000 g. The top layer was then transferred into a clean 10 mL screw top test tube. One mL of petroleum ether was added to the bottom layer. The tube was then capped and vortexed for 15 seconds and then centrifuged for 10 minutes at 1000 g. The top layer was then added to the previously removed top layer and 2 mL of deionized water was added to the combined top layers. The tubes were then capped and vortexed for 1 second and centrifuged for 5 minutes at 1000 g. The top layer was transferred to a conical gas chromatography vial, being careful not to include any of the bottom layers. The sample was then dried at 30°C with a nitrogen evaporator. Once evaporated, 100 µL of hexane was added and the vials were capped and analyzed by gas chromatography.

Gas chromatography

Methylated samples were separated on a Varian WCOT Fused Silica CP-SELECT FAME column (100 m × 0.25 mm diameter and 0.25 μm film thickness; Varian Canada Inc., Mississauga, Ontario) using a Varian 450 GC with FID. The column was operated at 100°C for 2 min, the temperature was raised to 175°C at 25°C/min, held for 30 min, raised again to 220°C at 15°C/min, held for 10 min, raised again to 240°C at 20°C/min, and held for 11 min. Total run time was 60 min, and samples were run with a 20:1 split ratio and column flow of 0.8 mL/min.

Calculations

Fatty acids were identified through the presence of peaks at different holding temperatures and subsequent time points relative the fatty acid standard. The data on the chromatogram was expressed as the AUC of the peak. The AUC of the peaks for the internal standards (C:15 and C:17) and BHT were removed to determine the AUC for fatty acids in the samples. The AUC of the peaks were recalculated and expressed as a percentage of the total area for g fatty acid/100 g of fatty acids.

Hepatic lipid droplet assessment

Liver frozen in OCT compound (Sakura Finetek, Torrance, CA, cat # 25608-930) was used to prepare 7 μm thick sections with a cryotome (Leica CM1850 uV, Wetzlar, Germany). The sections were stained with Oil Red O, a fat soluble dye, to visualize hepatic lipid droplets.

An Oil Red O stock solution was made by combining 0.7 g of Oil Red O (Sigma-Aldrich, St. Louis, MO cat # O0625) with 200 mL of 60% isopropanol. The mixture was

stirred overnight, filtered through a 0.2 µm filter and stored at 4°C. An Oil Red O working solution was made by combining 120 mL of stock solution with 80 mL of ddH_2O.The prepared sections were placed in 60% isopropanol for 5 minutes and then placed in an Oil Red O working solution for 30 minutes. The slides were then rinsed in 60% isopropanol and dried at room temperature.

Sections were visualized with a Zeiss Axioskop 2 plus microscope (Zeiss, Thornwood, NY) and images were captured with a Zeiss Axiocam digital camera using Axio Vision 4.6 (Zeiss, Thornwood, NY). Quantification of lipid droplet number and size was carried out using ImageJ software (National Insitute of Health, Bethesda, MD). For lipid droplet size measurements, 25 adjacent lipid droplets were randomly selected from 4 different sections of liver tissue for a total of 100 lipid droplets for each rat. For quantification of lipid droplet number, liver sections were divided into 0.01 mm^2 squares and all lipid droplets within this area were counted.

Western blotting

Principle

SDS-PAGE is a technique used to separate proteins based on their molecular weight. Total protein was extracted from the frozen liver tissue and the Pierce bicinchoninic acid (BCA) assay kit was used to quantify the protein concentration of the hepatic lysates though a colormetric assay. Based on the protein assay, protein lysates containing an equal amount of protein were heated to boiling to denature the protein and then loaded onto a polyacrylamide gel. An electrical current was used to pull the negatively charged proteins through the gel. The molecular weight of the protein

determines how fast it is pulled through the gel; smaller, lower molecular weight proteins travel faster and further than large molecular weight proteins which are slowed down by pore size. Once the proteins were separated by their molecular weights via SDS-PAGE they were then transferred to a polyvinylidene difluoride (PVDF) membrane to be probed with primary antibodies. A secondary antibody and chemiluminescent substrate were used to identify the location and relative amount of the primary antibody (ie: protein of interest). The relative intensity of the chemiluminescent reaction was imaged on a FluorCHEM® Q Western Blot imager, and quantified using AlphaView SA software (Protein Simple, Santa Clara, CA, USA).

Hepatic protein extraction

To quantify the amount of protein in the liver and make it available for Western blot analysis, the protein must be released from the appropriate tissue. This was done by grinding up 40 mg of liver into a fine powder under liquid nitrogen with a mortor and pestel. Next, 30 µL of 3× sample buffer (0.2 M Tris-HCl [pH 6.8], 3% SDS and 30% glycerol) per mg of liver was mixed with the powdered tissue. The mixture was set aside for a minimum of 15 minutes at room temperature to permit lysis of the cells. Next, the mixture was pipetted into 1.5 mL microfuge tubes and centrifuged for 20 minutes at 9400 g. The supernatant containing the protein was pipetted into a new microfuge tube and the remaining scum and pellet were discarded. The supernatant, containing the protein was then sonicated (Sonic dismembrator, Model 100, Thermo Fisher Scientific Inc., Ottawa, ON) for a minimum of 10 seconds and stored at -80^{o}C until used.

Protein quantification

The Pierce bicinchoninic acid (BCA) assay kit (Thermo Scientific, Rockford, IL,Cat. # 23225) was used to quantify the protein concentration of the hepatic protein extract. The assay is a colormetric assay using BCA to quantify the total protein content of a sample. In an alkaline medium, Cu^{+2} is reduced to Cu^{+1} by protein. Two molecules of BCA chelate with one Cu^{+1} to produce a purple pigment, which is proportionate to the amount of protein present in the sample. The purple color is chelated BCA-Cu^{+1}, which can be measured at an absorbance of 550 nm to determine the amount of protein per sample relative to a standard curve.

Reagents

1. BCA™ Protein Assay Reagent A (Thermo Scientific, Rockford, IL, prod # 23223): contains sodium carbonate, sodium bicarbonate, BCA™ detection reagent, and sodium tartate in 0.1 sodium hydroxide.
2. BCA™ Protein Assay Reagent B (Thermo Scientific, Rockford, IL, prod # 23224).
3. Albumin Standard (Thermo Scientific, Rockford, IL, prod. # 23209): contains 2.0 mg/mL bovine serum albumin in 0.9% aqueous NaCl solution containing sodium azide. A standard curve was prepared using 2.0 mg/mL bovine serum (BSA)(cat # 23209, Thermo Scientific, Rockford, IL, USA) diluted with ddH_2O to create concentations of 0.2, 0.4, 0.6, 0.8 and 1.0 mg/mL BSA, with ddH_2O acting as a blank.

Procedure

1. 10 µL of each standard or sample was added in triplicate into a 96-well plate (cat # 167008, NUNC^{TM}, Roskilde, Denmark).
2. The reaction reagent mixture was mixed by adding the appropriate amount (1:50) of BCA Reagent B to BCA Reagent A.
3. 200 µL of reaction mixture was added to each well using a multichannel pipette.
4. The plate was then covered with a plate lid and sealed with parafilm.
5. The plate, containing the samples, was then incubated at 37^{o}C for 30 minutes.
6. The plate was removed from the incubator and any bubbles present were popped by expelling air from an empty pipette tip.
7. Plates were then read on the FLUOstar Omega plate reader (BMG LABTECH, Ortenberg, Germany) at an absorbance of 550 nm.

Sodium dodecysulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

Reagents

% Acrylamide gel	**20% Acrylamide (mL)**	**1.5 M Tris-HCl pH 8.8 (mL)**	**ddH_2O (mL)**	**10% APS (µL)**	**10% SDS (µL)**	**TEMED (µL)**
5% Separating gel	5	4.5	12.9	200	200	16
10% Separating gel	10	4.5	5.3	200	200	16
15% Separating gel	15	4.5	0.3	200	200	16

% Acrylamide gel	20% Acrylamide (mL)	1.5 M Tris-HCl pH 8.8 (mL)	ddH_2O (mL)	10% APS (µL)	10% SDS (µL)	TEMED (µL)
5% Stacking Gel	2	2	3.75	80	80	20

APS = ammonium persulfate, SDS = Sodium dodecyl sulphate, TEMED = N,N,N',N'-tetramethylethylenediamine (MP Biomedicals LLC, Solon OH, cat. # 805615)

Other reagents

- t-amyl alcohol
- 10% bromophenol blue (w/v)(BPB)
- 2- mercaptoethanol (β-ME)(14.16 M)
- 5× running buffer: 200 mL 10% SDS, 288 g glycine, 60.6 g Tris-base, top up to 4 L with ddH_2O
- 5× transfer buffer: 75.75 g Tris-base, 242.5 g glycine, top up to 4 L with ddH_2O
- Methanol
- 5× Tris-buffered saline with Tween-20 (TBS-T): 600 mL 5M NaCl, 40 mL 1 M Tris-HCl pH 7.4, 10 mL Tween-20, ddH_2O to yield a final volume of 4 L

Procedure

To prepare the separating gel, 2 glass plates and 2 spacers were cleaned with ddH_2O. The gasket was wrapped around the bottom of one of the glass plates and the plates were sandwiched together with casting clips. The separating gel was prepared accordingly and pipetted into the space between the two glass plates. A 50-well comb was added and t-amyl alcohol was pipetted onto the top of the gel to remove any air bubbles present. The gel was allowed to polymerize for 45 minutes. After polymerization

the t-amyl alcohol was rinsed off with ddH_2O. The stacking gel was then prepared accordingly and pipetted down the sides of the comb to create the wells. The stacking gel was then allowed to polymerize for 10 minutes.

While the stacking gel was polymerizing, 10 µL of the respective protein samples were prepared by aliquoting the calculated protein amount with 3× sample buffer. The calculation to determine the amount of sample buffer to add to obtain the desired protein amount was as follows:

$$10\ \mu L - \frac{amount\ of\ protein\ to\ load\ onto\ gel\ (\mu g)}{protein\ concentation\ from\ protein\ assay\ (\frac{\mu g}{mL})}$$

A ratio of 1:1 10% BPB and β-ME was prepared and 1 µL of the mixture was added to 10 µL of the calculated protein sample with 3× sample buffer. The samples were then placed into a water bath and microwaved on high for 3 minutes to denature the proteins.

Once the stacking gel had polymerized, the casting clamps were removed and the gasket was peeled off. Half of the working running buffer solution (200 mL 5× running buffer and 800 mL ddH_2O) was poured onto the bottom of the electrophoresis tank and the plates were gently placed into the tank. The plates were clamped to the electrophoresis apparatus and the comb was removed from the wells. The rest of the running buffer was poured onto the middle of the tank until each well was filled with buffer. A protein molecular weight marker was loaded into the 1st well and a well half way through the protein samples using a glass syringe. Samples were then loaded into each well. Once the samples were loaded, the electrophoresis tank was moved into the cold room (4^{o}C), and a glass hooked pipette was used to ensure all the bubbles were removed from the bottom of the tank. The electrophoresis tank was then connected to a

power supply and run at 70 mA for about 1.5 hours, or until the dye front reached the bottom of the gel.

After the gel was done running, the gel was removed from the tank and the glass plates were pried apart. The stacking gel was removed from the separating gel and discarded. A sheet of blotting paper was placed on top of the gel and the gel was carefully removed from the glass plate. A PVDF membrane was then labelled and dipped into methanol for 1 minute before being placed onto the gel. Another piece of blotting paper was then placed on top of the PVDF membrane. Bubbles were removed using a roller. The sandwich was placed between 2 fibre board pads and placed into the transfer gel cassette within a container filled with the working transfer solution (500 mL methanol, 400 mL 5× transfer buffer, topped up with 3 L ddH_2O). The cassette was placed into the transfer tank and filled with the working transfer buffer. The transfer tank was then moved to the cold room (4^oC) and a stir bar was added. The gel was allowed to transfer on a stir plate for 45 minutes at 100 V. Once the transfer was complete, the PVDF membrane was placed into a container filled with 1× TBST for storage until probed with antibodies.

Immunoblotting

Immunostaining was used to determine the relative amount of several proteins in a particular sample. This procedure was used to determine the changes in the factors that lead to hepatic fatty acid oxidation (pPPARα, PPARα and ACO), hepatic fatty acid synthesis (SREBP1, pACC and ACC), hepatic fatty acid uptake (HIG2) and glucose uptake in the liver (FGF-21 and PTP1B). This procedure works in 3 steps, with the first

step being the incubation with a primary antibody. The second step is incubation with a secondary antibody, which is complexed to horseradish peroxidase (HRP). The secondary antibody will bind to the initial primary antibody applied. The third step is the detection of the labelled proteins. When a chemiluminescent substrate is added it causes the HRP to catalyze a reaction that results in the emission of light.

Reagents

- 3% BSA in TBST
- 5× TBST: 600 mL 5 M NaCl, 40 mL 1 M Tris-HCl pH 7.4, 10 mL Tween-20, ddH2O to yield a final volume of 4 L
- 1% BSA- TBST
- Primary antibody (see Table 8)
- Secondary antibody (see Table 8)
- Amersham ™ ECL ™ Prime Western Blotting Detection Reagent

Procedure

Membranes were blocked for 1 hour in 3% BSA-TBST and washed 4 times for 5 minutes in 1× TBST. All primary antibodies were run in 3% BSA-TBST for 1 hour, and again washed 4 times for 5 minutes in 1× TBST before the secondary antibody was added. The secondary antibody was 1:10000 anti-rabbit in 1% BSA-TBST (unless otherwise specified). Membranes were then dipped in Amersham ™ ECL ™ Prime Western Blotting Detection Reagent (GE Healthcare, Little Chalfont, UK) and imaged on a FluorCHEM® Q Western Blot imager, and quantified using AlphaView SA software (Protein Simple, Santa Clara, CA, USA). In AlphaView, multiple band analysis was selected and in “Bkground”, local background was selected. Region tools were used to

select bands of interest and BC average was exported into a Microsoft Excel file for further analysis. The calculations were done by taking the ratio of the BC average of the antibody to the BC average of the loading control and expressed as arbitrary units.

Stripping of membrane blots

Membranes were stripped after Western blotting to allow the membrane to be probed with other antibodies with similar molecular weights.

Reagents

- Stripping buffer: 100 mL 10% SDS, 62.5 mL 0.5 M Tris HCl pH 6.8 and ddH_2O to yield a final volume of 500 mL
- 2-mercaptoethanol (β-ME)
- 1× TBST
- 10% bleach

The membrane was covered with 50 mL of stripping buffer and, in the fume hood, 400 µL of β-ME was added. The container was closed and placed into a larger container and sealed with paraffin tape. This was done to reduce the amount of odour. The boxes were placed on the shaker overnight and the stripping buffer solution was poured down the sink in the fume hood the next morning. The membranes were then rinsed 4 times with 1× TBST for 5 minutes each rinse. Once rinsed the membranes were stored for subsequent use in a container containing 1× TBST. The old containers were rinsed with 10% bleach and left in the fume hood until the odour dissipated.

Table 8: Antibodies used in Western Blotting analysis

Primary Antibody	Source	Primary dilution and incubation time	Secondary antibody	Secondary dilution and incubation time	Molecular weight (kDa)	% Separating gel
FGF-21 (H-105)	Santa Cruz Cat # sc-292879	1:200, 1 hour	anti-rabbit HRP[1]	1:10000, 1 hour	18-20	15
PTP-1B	Cell Signalling Cat # 5311	1:1000, 1 hour	anti-rabbit HRP	1:10000, 1 hour	50	10
SREBP-1c (C-20)	Santa Cruz Cat # 366	1:500, 1 hour	anti-rabbit HRP	1:10000, 1 hour	68/125	10
pACC	Cell Signalling Cat # 3661s	1:1000, 1 hour	anti-rabbit HRP	1:10000, 1 hour	280	5
ACC	Cell Signalling Cat # 4190	1:1000, 1 hour	anti-rabbit HRP	1:10000, 1 hour	265	5
ACO	Santa Cruz Cat # sc-98499	1:100, 1 hour	anti-rabbit HRP	1:10000, 1 hour	74	10
pPPARα	Abcam Cat #3484	1:500, 1 hour	anti-rabbit HRP	1:10000, 1 hour	52	15
PPARα	Abcam Cat # 2774	1:1000, 1 hour	anti-mouse HRP	1:10000, 1 hour	52	15
HIG2 (C-14)	Santa Cruz Cat # sc-137518	1:500, 1 hour	anti-rabbit HRP	1:10000, 1 hour	7	15

β-Tubulin[3]	Cell Signaling Cat # 2146	1:1000, 1 hour	anti-rabbit HRP	1:10000, 1 hour	55	10-15
eEF2[23]	Cell Signaling Cat # 2332	1:1000, 1 hour	anti-rabbit HRP	1:10000, 1 hour	95	5

[1]HRP= Horseradish peroxidase
[2]eEF2= Eukaryotic translation elongation factor 2
[3]Loading control

Adipocyte Size

Frozen sections of epididymal adipose tissue were used to determine adipocyte size and distribution of small and large adipocytes between genotypes and diet interventions. Epididymal adipose tissue frozen in cryogenic gel embedding medium was used to prepare the slides. Ten µm sections of adipose tissue were prepared with a cryotome (Leica CM1850 uV, Wetzlar, Germany). The sections were placed on microscope slides and analyzed unstained. Images of the tissue and a micrometer were captured with a light microscope fitted with a camera at 200× magnification using Axio Vision 4.6 (Zeiss, Thornwood, NY). Cell areas (μm^2) of 125 adipocytes per treatment group were measured with ImageJ (National Insitute of Health, Bethesda, MD). The area of a circle was imposed on an image of a 0.01 mm micrometer in Image J and it was used to calculate a conversion factor to convert arbitrary units to squared micrometers as the unit of adipocyte size.

Statistical analysis

One way ANOVA was used for endpoint data while repeated measures ANOVA was used for time course data, such as feed intake, weekly body weights or glucose concentrations during OGTT (SAS Version 9.2, SAS institute, Cary, NC). Following ANOVA, means testing with Duncan's multiple range test was performed. If the data were not normal or homogeneous, data were analyzed after log transformation, or non-parametric testing was used. Non–parametric testing used the Kruskal-Wallis test, followed by least significant difference post hoc testing with Tukey correction for multiple comparisons. Pearson (normal data) or Spearman's (non-normal data) correlations were used to determine the relationship between the two sets of data. The Chi-squared test was used for liver lipid size and adipocyte size distribution. Outliers (± 2.5 standard deviations from the mean) were removed from the data set before analysis. The data are reported as means ± the standard error of the mean and differences were considered significant at $p<0.05$.

RESULTS

Feed intake and physical characteristics

Feed intake

Throughout the study the faLA consumed more feed than the faEPA and faDHA on all weeks except for week 3, where all the *fa/fa* groups consumed similar amounts of feed (Figure 3A). Oddly, the faEPA and faDHA groups ate less feed at week 1; compared to the faLA and faALA they consumed increasingly more feed at week 2. This might be explained by the high concentration of fish oil present affecting the initial palatability of the diet. In relation to total feed intake the faLA and faALA group consumed the highest amount of total feed, which was about 10% higher than the faEPA and faDHA groups which were similar among each other (Table 9). The feed efficacy ratio is an indicator of how well food mass was converted into body mass and feed efficacy ratio was calculated as total weight gain (g)/ total feed intake (g) (Table 9). A higher ratio means that food mass was converted into body mass more effectively. Interestingly, the lnLA group had a higher feed efficacy ratio than the *fa/fa* experimental groups, where no differences among the groups were seen (Table 9).

Body weight

The weekly body weights of the groups were generally parallel to weekly feed intake where faLA had higher body weights than faEPA and faDHA at weeks 2 through 8 (Figure 3B). The faEPA and faDHA groups had similar body weights throughout the study. At the end of the 8 week study faLA and faALA had the highest final body weight overall. There was no difference between the faEPA and faDHA groups in relation to

final weight, which was lower than faLA and faALA but not as low as lnLA. When comparing to the faBASE, the faLA and faALA weighed approximately 2.6 times more and the lnLA weighed about 2 times more.

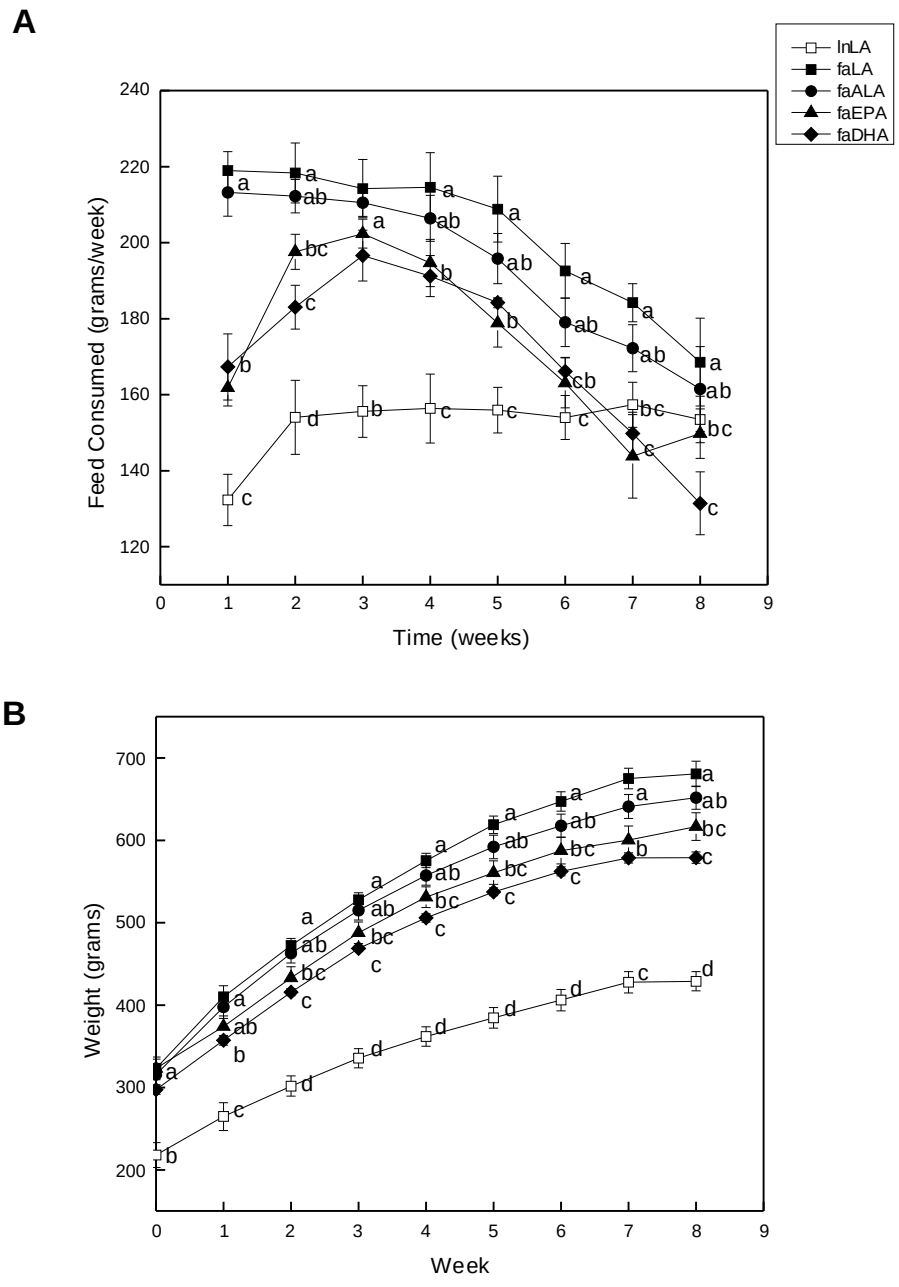


Figure 3: Weekly feed intake (A) and weekly body weight (B). Weekly feed intake and body weight are presented as means ± SEM, n=10 except for faLA, faALA and faDHA which were n=8, 9 and 9, respectively. Different letters indicate statistical significance ($p<0.05$) among the means at the same time point.

Table 9: Body weight and feed intake

	lnLA	faLA	faALA	faEPA	faDHA	faBASE
Total Feed Intake (g)	1219 ± 48^{c}	1620 ± 38^{a}	1551 ± 27^{a}	1392 ± 31^{b}	1370 ± 76^{b}	---
Feed Efficacy Ratio (g total feed/g weight gain)	5.80 ± 0.25^{a}	4.59 ± 0.16^{b}	4.65 ± 0.13^{b}	4.80 ± 0.14^{b}	4.92 ± 0.2^{b}	----
Initial body weight (g)	218 ± 6^{b}	323 ± 15^{a}	316 ± 11^{a}	323 ± 12^{a}	297 ± 13^{a}	----
Final Body Weight (g)	429 ± 7^{c}	689 ± 12^{a}	652 ± 15^{a}	616 ± 14^{b}	579 ± 17^{b}	247 ± 4^{d}
Total Weight Gain (g)[1]	211 ± 7^{d}	357 ± 19^{a}	336 ± 13^{ab}	293 ± 14^{bc}	282 ± 13^{c}	----

Values are expressed as means ± SEM, n =10 except for faLA, faALA and faEPA where n= 8, 9 and 9, respectively. Different superscripts indicate significant differences ($p<0.05$) among the means and an absence of superscripts indicates no significant differences.

[1]Data were analyzed using non-parametric testing for statistical analysis.

Tissue weight

Fat pads

Fat pad and body weights were used as indicators of obesity. Total body fat was measured by whole body MRI using an EchoMRI-700™ whole body QMR instrument (Echo Medical Systems, Houston TX). The results were corrected to body weight (Table 10). For absolute fat pad mass see Appendix 1. All the *fa/fa* experimental animals had similar total body fat, and the lnLA group had the least amount of body fat. Although all *fa/fa* animals had more total fat than the lnLA group, there were differences seen in the weights of the individual adipose depots. Total visceral fat was determined by the sum of the epididymal, perirenal and mesenteric fat. There were no differences between the *fa/fa* experimental groups in relation to visceral fat, although faDHA was also similar to faBASE. The lnLA group had the lowest visceral fat. The faLA group had the highest epididymal fat, which was similar to the group fed the faEPA diet, but greater than the faALA, faDHA and faBASE groups, which were similar amongst each other. Again, the lnLA group had the lowest epididymal fat. There was no difference among the *fa/fa* experimental groups in relation to perirenal fat. The faALA group had the most mesenteric fat, which was similar to faBASE and faEPA, but greater than faDHA. None were as low as the lnLA group. Subcutaneous fat, which was determined by subtracting total visceral fat from total fat, was highest in the faLA group. Subcutaneous fat of the faEPA group was similar to the faDHA and faALA group, but greater than the faBASE group. None were as low as the lnLA group.

Liver weight

Liver weight is expressed as percent body weight. For a complete list of absolute tissue weight see Appendix 1. Liver weight, expressed as percent body weight did not differ among *fa/fa* groups (Table 10). It was also found that the livers of the lnLA group weighed significantly less than its *fa/fa* counterparts fed the same diet.

Table 10: Fat pads and liver weight

	lnLA	faLA	faALA	faEPA	faDHA	faBASE
Total Body Fat[1] (g/100 g bw)	12.4 ± 0.6^{c}	45.4 ± 1.1^{a}	46.7 ± 1.1^{a}	47.2 ± 0.7^{a}	44.7 ± 0.2^{ab}	40.1 ± 2.0^{b}
Total Visceral Fat[1] (g/100 g bw)	5.7 ± 0.4^{c}	9.9 ± 0.2^{a}	9.4 ± 0.3^{a}	9.0 ± 0.4^{a}	8.1 ± 0.1^{ab}	7.2 ± 0.2^{b}
Epididyimal Fat (g/100 g bw)	1.7 ± 0.1^{c}	2.8 ± 0.1^{a}	2.4 ± 0.1^{b}	2.5 ± 0.1^{ab}	2.4 ± 0.1^{b}	2.4 ± 0.1^{b}
Perirenal Fat[1] (g/100 g bw)	2.4 ± 0.4^{b}	4.7 ± 0.1^{a}	4.3 ± 0.2^{a}	4.1 ± 0.3^{a}	3.6 ± 0.1^{a}	2.4 ± 0.1^{b}
Mesenteric Fat (g/100 g bw)	1.6 ± 0.1^{c}	2.4 ± 0.1^{ab}	2.7 ± 0.1^{a}	2.4 ± 0.1^{ab}	2.1 ± 0.1^{b}	2.5 ± 0.1^{a}
Subcutaneous Fat[1] (g/100 g bw)	6.9 ± 0.7^{d}	44.0 ± 1.1^{a}	37.3 ± 1.0^{bc}	38.2 ± 0.8^{b}	36.6 ± 0.1^{bc}	32.9 ± 1.9^{c}
Liver weight (g/100 g bw)	2.9 ± 0.1^{b}	4.7 ± 0.4^{a}	5.2 ± 0.2^{a}	4.8 ± 0.2^{a}	4.6 ± 0.2^{a}	4.5 ± 0.1^{a}

Values are expressed as means ± SEM, n =10 except for faLA, faALA and faEPA where n= 8, 9 and 9 respectively. Different superscript letters indicate significant differences ($p<0.05$) among the means. Total body fat was determined using whole body MRI, visceral fat included epididyimal, perirenal and mesenteric fat pads, and subcutaneous fat was determined by subtracting visceral fat from total fat.

[1]Data were analyzed using non-parametric testing for statistical analysis.

Blood pressure

The faBASE group had lower systolic and diastolic pressure and mean arteriole pressure (MAP) than all the other groups (Table 11). The faDHA group had lower diastolic blood pressure compared to the lnLA and faALA groups.

Table 11: Blood pressure and MAP

	lnLA	**faLA**	**faALA**	**faEPA**	**faDHA**	**faBASE**
Systolic (mm/Hg)	143 ± 8^{a}	136 ± 16^{a}	141 ± 19^{a}	129 ± 13^{a}	131 ± 12^{a}	100 ± 10^{b}
Diastolic (mm/Hg)	102 ± 9^{a}	91 ± 12^{ab}	99 ± 17^{a}	91 ± 12^{ab}	84 ± 14^{b}	70 ± 7^{c}
MAP (mm/Hg)	115 ± 9^{a}	105 ± 13^{a}	113 ± 17^{a}	103 ± 12^{a}	99 ± 13^{a}	80 ± 8^{b}

Values are expressed as means ± SEM, n =10 except for faLA, faALA and faEPA where n=8, 9 and 9 respectively. Different superscript letters indicate significant differences ($p<0.05$) among the means.

Glycemia and lipidemia

Serum glucose and insulin

After a 5 hour fast in metabolic cages, there were no differences seen in fasting serum glucose among the experimental groups at the end of the 8 week study or baseline animals (Figure 4A). All *fa/fa* groups had higher serum glucose than the lnLA group. Fasting serum insulin was similar between the faALA and faEPA groups and they were higher than the faLA and faDHA groups, which were similar amongst each other. None were as low as the lnLA group, or the faBASE group (Figure 4B).

OGTT

By 15 minutes (t15) of the OGTT, the lnLA group had peaked and it had the highest serum glucose, which was similar to faBASE, faALA and lnLA at t15 (Figure 5A). The lnLA group had a serum glucose concentration that was 2-3 mmol/L higher than faDHA, which had the lowest serum glucose at t15 and, which was similar to faEPA. By t30, the faALA group had the highest concentration of serum glucose, which was similar to lnLA, faLA and faEPA. The faBASE group had the lowest serum glucose at t30, which was simiar to faDHA. At t60, the faALA still had the highest concentration of serum glucose, which was slightly lower than faALA at t30. The serum concentration of faALA at t60 was similar to faEPA, faDHA and lnLA. By t60, faLA had a lower serum glucose concentration than faALA, which was similar to faEPA, faDHA and lnLA, but not as low as faBASE. By t60, the serum glucose concentration of faBASE was similar to t0 at approximately 7 mmol/L, while faALA was approximately 11 mmol/L. At t120, faDHA had the highest serum glucose concentration, which was similar to faEPA and

faALA. The faBASE group had the lowest serum glucose by t120, which was similar to the lnLA and faLA. At t120 the faDHA and faBASE had a difference in serum glucose levels of approximately 3 mmol/L. While changes were seen through the time course of the OGTT, there were no differences seen in the AUC for glucose among any of the *fa/fa* experimental animals, which were all significantly higher than the lnLA and faBASE animals (Figure 6A).

After a glucose load, the faALA and faEPA had similar insulin concentrations from t0 to t120, while faLA and faDHA had lower circulating insulin than faALA (Figure 5B). None of the experimental diets provided to the *fa/fa* Zucker rats was able to reduce circulating insulin to the same level as the faBASE and lnLA, which were 5 to 8 fold lower than the *fa/fa* experimental groups. The AUC for insulin was lower in the faDHA and faLA groups compared to faALA, while faALA was similar to faEPA (Figure 6B). None of the treatment groups had AUCs as low as the lnLA or the faBASE groups. The HOMA-IR scores were elevated in faALA and faEPA compared to faLA but not faDHA (Figure 6C). The faLA had the lowest HOMA-IR score of the experimental diets, but none were as low as the lnLA or faBASE groups.

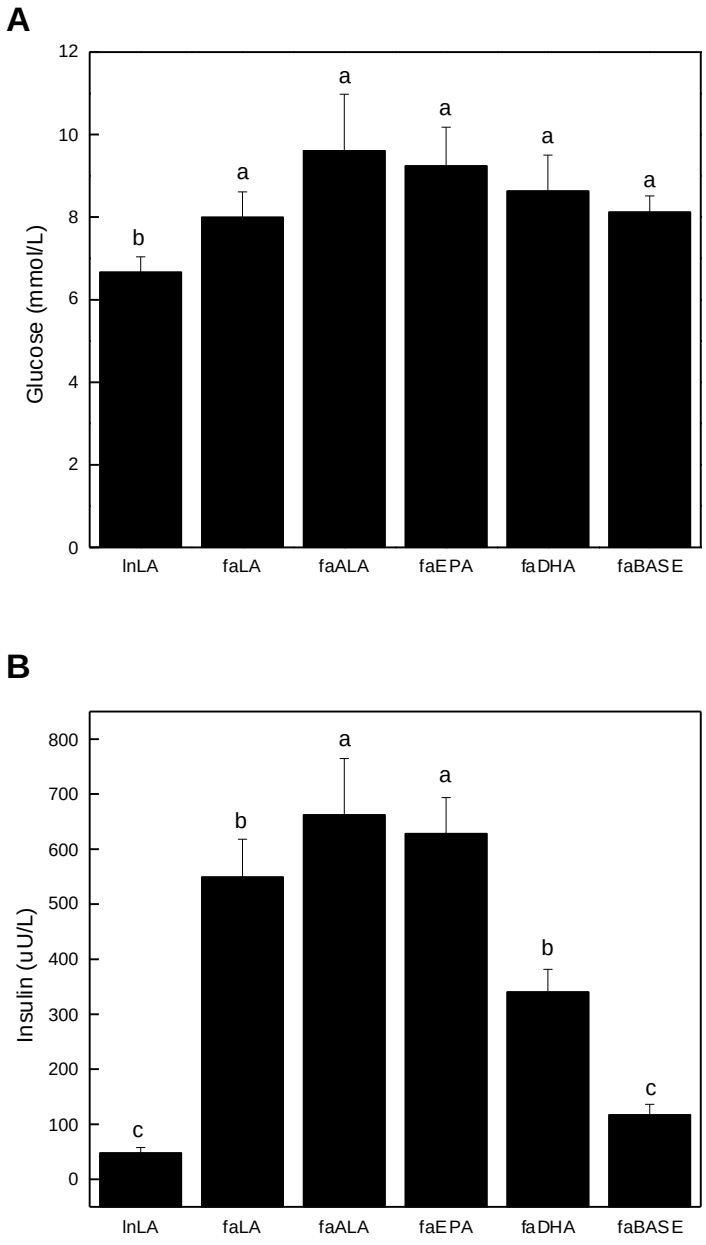


Figure 4: Fasting serum glucose (A) and insulin (B). Values are expressed as means ± SEM with n =6-10. Different superscript letters indicate significant differences ($p<0.05$) among the means.

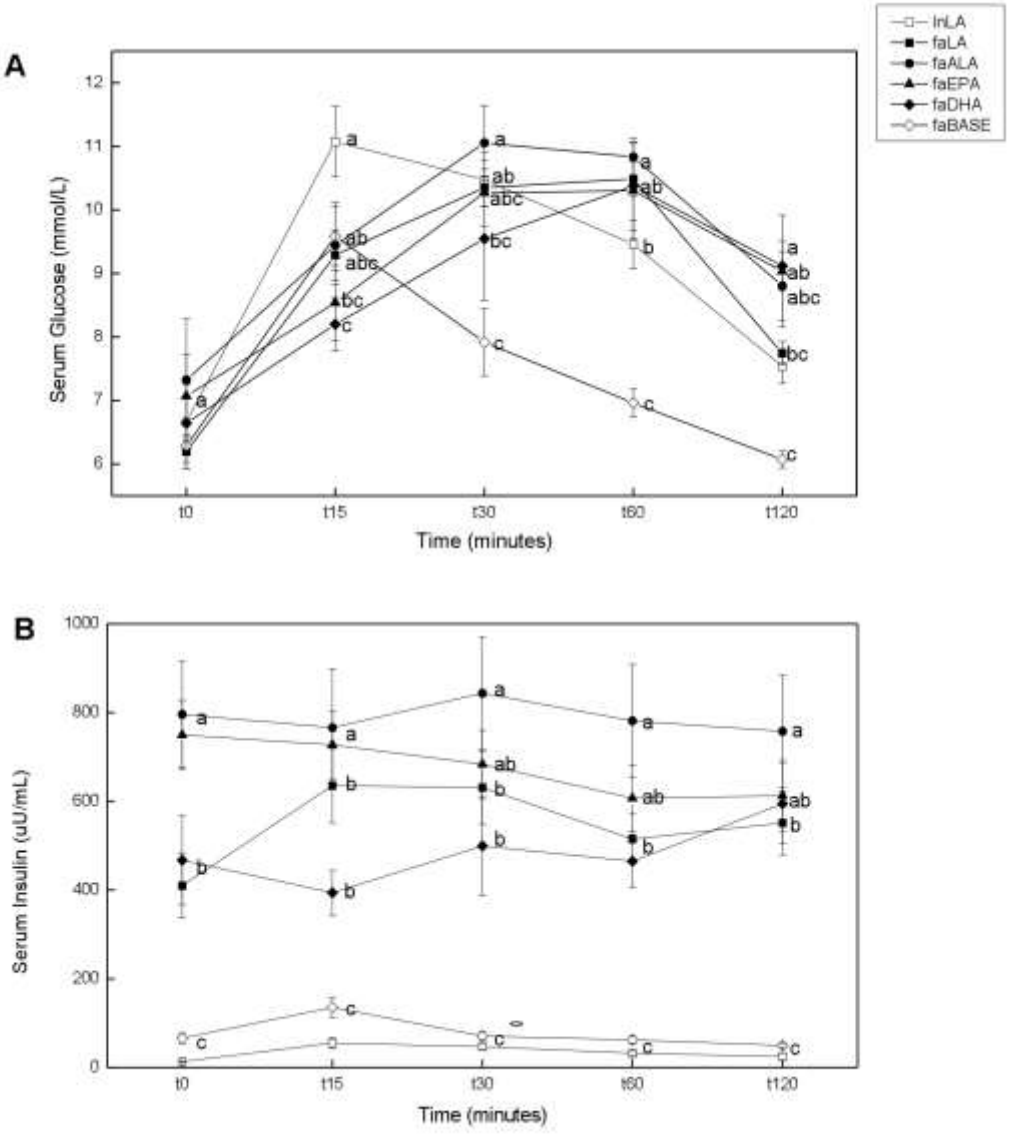


Figure 5: OGTT. Blood glucose at various time points (A) and blood insulin at various time points (B). Data are presented as mean ± SEM, n=8-10. Different letters indicate statistical significance ($p<0.05$) among the means at the same time point.

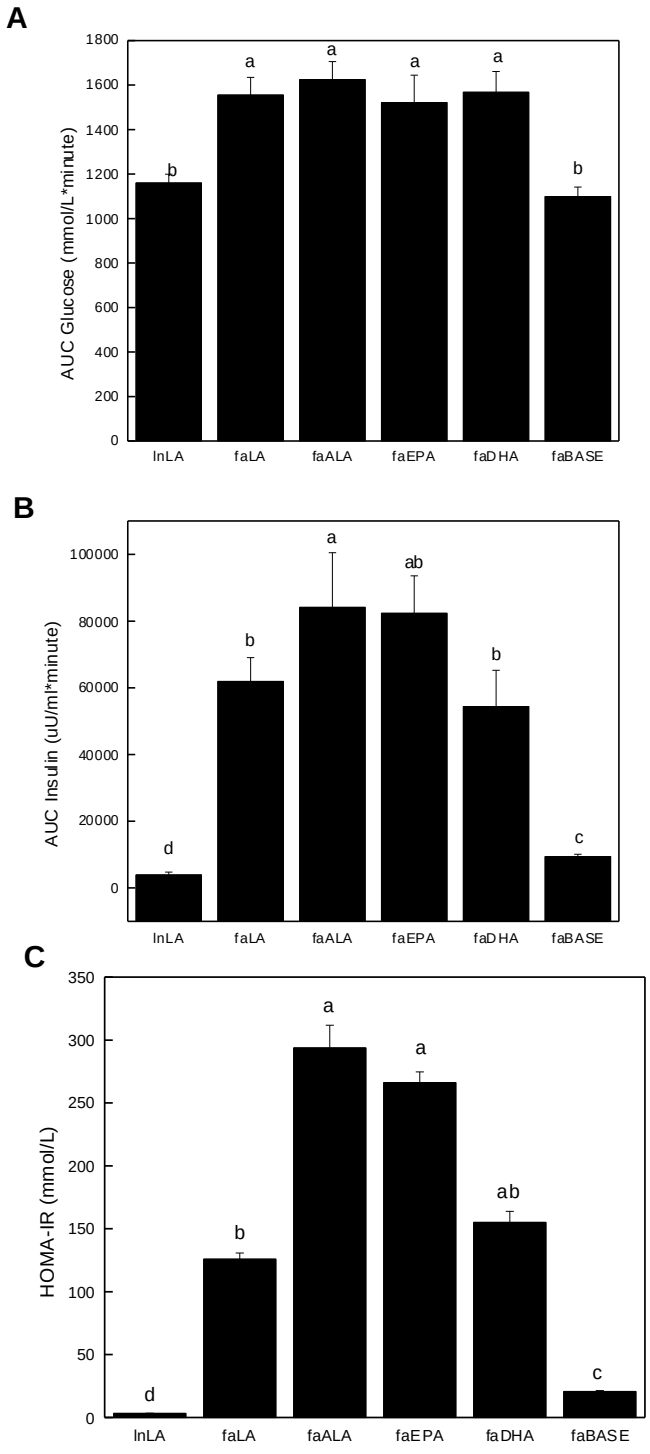


Figure 6: $AUC_{glucose}$ (A), $AUC_{insulin}$ (B) and HOMA-IR (C). Values are expressed as means ± SEM with n =6-10. Different superscripts indicate significant differences ($p<0.05$) among the means.

Molecular markers of impaired glucose tolerance and insulin resistance

FGF-21 and PTP1B in the liver are correlated with impaired glucose tolerance and insulin sensitivity (Woo et al., 2013; Tamrakar et al., 2014). FGF-21 and PTP1B were analyzed in liver samples by Western blotting and relative intensities of the bands were quantified using AlphaView SA software (Protein Simple, Santa Clara, CA, USA). There was no detectable amount of FGF-21 in the protein samples from the liver. There were no differences seen in PTP1B amongst any of the groups (Table 12 and Figure 7).

Table 12: Molecular markers of glucose uptake in the liver

	lnLA	**faLA**	**faALA**	**faEPA**	**faDHA**	**faBASE**
FGF-21 (H-105)	ND	ND	ND	ND	ND	ND
PTP-1B	0.9 ± 0.1	1.5 ± 0.3	1.7 ± 0.4	1.4 ± 0.2	1.2 ± 0.2	1.6 ± 0.6

Values are expressed as means (arbitrary units relative to β-tubulin) ± SEM, n =6. Different superscript letters indicate significant differences ($p<0.05$) among the means and an absence of a superscript letter indicates no significant differences.

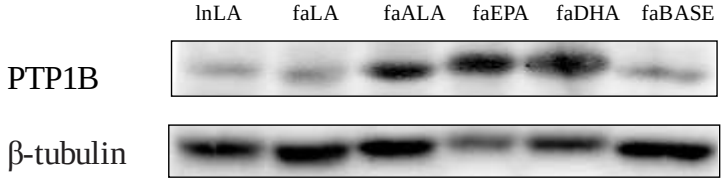


Figure 7: Representative blot of PTP1B. Relative levels of PTP1B were determined by Western blotting of liver

Lipid profile

The lipid profile was characterized by measuring 12 hour fasting serum for total cholesterol, HDL-C, LDL-C and TAG. The faLA and faALA had the highest total cholesterol, while all the other experimental groups had similar total cholesterol amongst each other. LDL-C was highest in the faLA group, followed by faALA, and both were significantly higher than the other groups which were similar amongst each other (Table 13). The faLA group also had the highest concentration of HDL-C followed by faALA, which were both significantly higher than the other groups. The faDHA had the lowest HDL-C. The faEPA had lower TAG than the faALA and faBASE while the lnLA had the lowest.

Table 13: Lipidemia

	lnLA	faLA	faALA	faEPA	faDHA	faBASE
Total Cholesterol (mmol/L)[1]	2.59 ± 0.10^{c}	7.02 ± 0.66^{a}	7.29 ± 1.28^{a}	3.45 ± 0.23^{bc}	2.79 ± 0.28^{bc}	4.33 ± 0.23^{bc}
LDL-C (mmol/L)	0.38 ± 0.03^{c}	1.56 ± 0.31^{a}	0.81 ± 0.28^{b}	0.25 ± 0.04^{c}	0.17 ± 0.06^{c}	0.22 ± 0.05^{c}
HDL-C (mmol/L)[2]	2.39 ± 0.06^{d}	5.05 ± 0.45^{a}	3.91 ± 0.48^{b}	2.36 ± 0.13^{cd}	1.72 ± 0.23^{e}	2.94 ± 0.22^{c}
TAG (mmol/L)	0.87 ± 0.09^{d}	4.66 ± 0.51^{abc}	5.66 ± 0.53^{a}	4.02 ± 0.51^{c}	4.76 ± 0.80^{bc}	4.81 ± 0.40^{ab}

Values are expressed as means ± SEM, n =10 except for faLA, faALA and faEPA where n= 8, 9 and 9, respectively. Different superscript letters indicate significant differences ($p<0.05$) among the means.
[1]Data were analyzed using non-parametric testing for statistical analysis.
[2]Data was log transformed prior to ANOVA.

Hepatic function

Total liver lipids and liver lipid droplet distribution

The lnLA group had the lowest percent liver lipids compared to all the other groups while faALA had the highest concentration of liver total lipids, except when compared to faLA (Figure 8A). The faDHA group had similar hepatic liver lipid concentrations as the faBASE group and was lower than the faLA and faALA groups. The faEPA group was lower than the faALA and not different from faDHA or faBASE.

There were no differences in the average size of liver lipid droplets among the *fa/fa* experimental groups (Figure 8B) despite 2 fold larger droplets in faLA vs faEPA and faDHA as shown in Figure 9. All lipid droplets of the *fa/fa* experimental groups were significantly larger than the lnLA and the faBASE groups, which were too small in size to quantify for area (Figure 10). Although there were no significant differences in relation to the average size of the lipid droplets, there was a trend in the distribution of the sizes of the lipid droplets. The faLA had more large lipid droplets compared to the other groups followed by faALA, faEPA and faDHA, respectively (Figure 8C). The faDHA group had 80% of its lipid droplets in the smallest size range (0-100 μm^2) compared to 60% for faEPA, 47% for faALA and 52% for faLA. Chi-squared testing confirmed that there were differences among the dietary groups for all the size ranges.

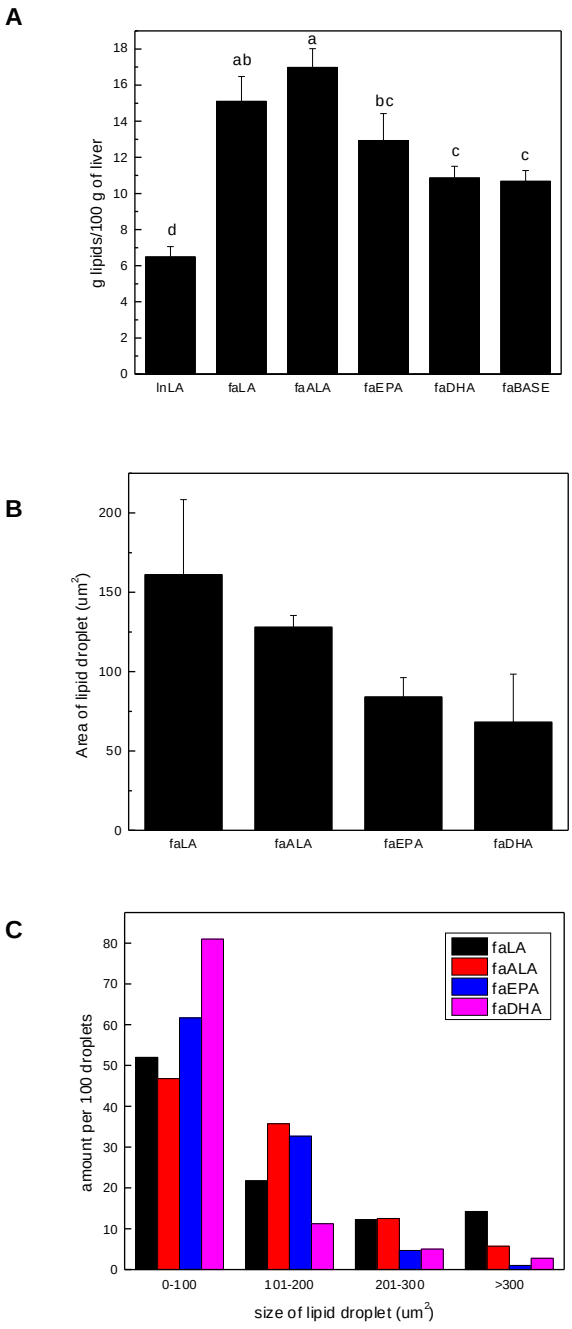


Figure 8: Liver total lipids (A), size of liver lipid droplets (B) and distribution of the sizes of lipid droplet in the liver (C). (A) Values are expressed as mean ± SEM, n = 6-7 for liver total lipids. Different letters indicate significant differences ($p<0.05$) among the means. (B) Values are expressed as means ± SEM, n =4. A p value of $p<0.05$ was used and an absence of letters indicates no significant differences. (C) Values are expressed as number per 100 droplets.

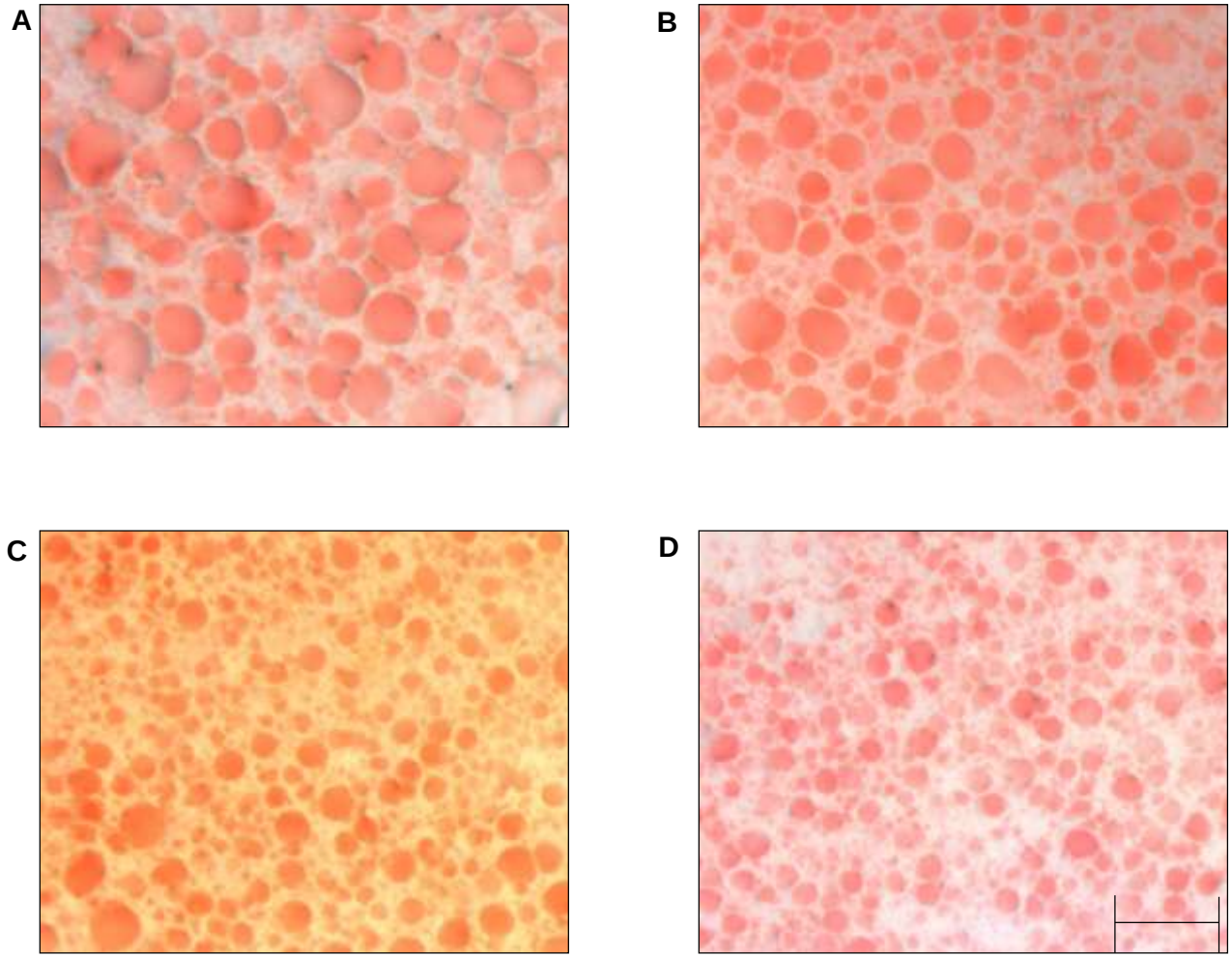


Figure 9: Liver lipid droplets for faLA (A), faALA (B), faEPA (C), faDHA (D). Representative images of liver droplets identified by Oil Red O. Scale bar equal to 0.05 mm.

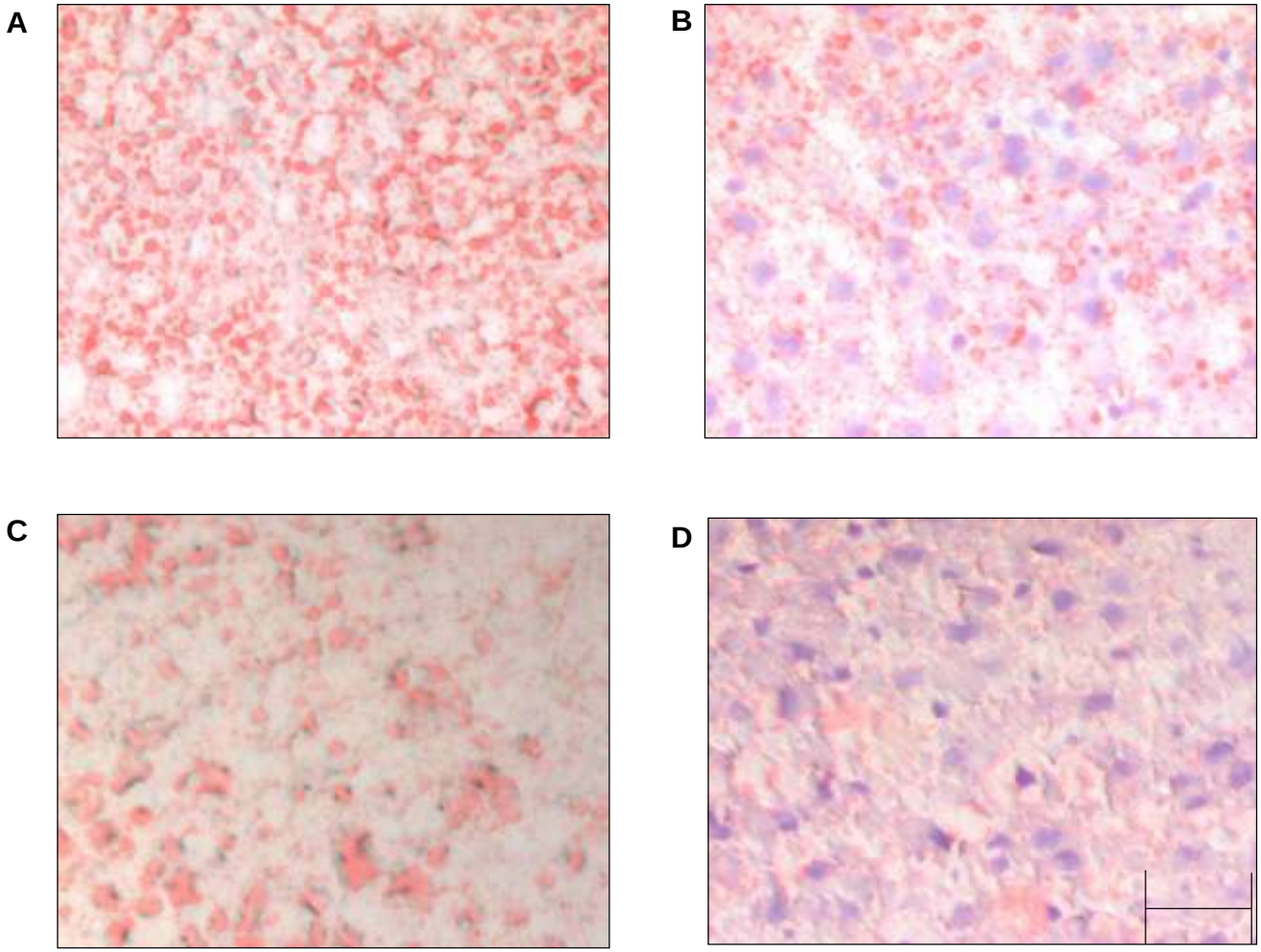


Figure 10: Liver lipid droplets for lnLA (A and B) and faBASE (C and D). Representative images of liver droplets identified by Oil Red O (A and C) and counter stained with hematoxylin (B and D) Scale bar equal to 0.05 mm

Hepatic fatty acid composition

Hepatic TAG

The lnLA group had the lowest proportion of SFA in the hepatic TAG fatty acid composition compared to all the other groups, but it was similar to the faDHA groups (Table 14). The faLA group had similar hepatic SFA as the faALA, which were also similar to the faBASE. The faEPA group had an intermediate proportion of SFA which was not different from faDHA. In regards to total MUFA, the lnLA had less MUFA than faLA. The faLA group had similar MUFA as the faEPA, faDHA and faBASE group, but the faBASE was higher compared to the faEPA or faDHA. The faALA group had the highest total MUFA in the hepatic TAG fraction. In terms of total PUFA, lnLA had the highest total PUFA, while the faALA had the lowest amount, which was similar to faLA. The faBASE group had the lowest proportion PUFA in its hepatic TAG fraction but was similar to faALA. While lnLA had the highest total PUFA it also had a low proportion of total n3, which was similar to the faLA and faBASE groups. Furthermore, faDHA had the highest total n3 PUFA in its hepatic TAG fraction while faALA had 2-fold less, which was similar to faEPA. The lnLA group had the highest total n6, while the faALA had the lowest, which was similar to faDHA and faBASE. The faLA group was similar to faEPA, which was also similar to faDHA. The faLA group had the highest n6/n3 ratio, while lnLA came next (Table 14). There were no differences in the remaining groups in relation to n6/n3 ratio, which ranged from 0.7-7.2.

In general, the n3 fatty acid profile of the diet was reflected in the n3 fatty acid profile of hepatic TAG. The faALA group had the highest ALA (C18:3n3), while the

faLA and lnLA and faBASE had the least (Figure 11A). The faEPA group had the highest EPA (C20:5n3) while faLA had the least, which was similar to lnLA and faBASE (Figure 11B). The faDHA group had the second highest EPA followed by faALA, which was similar to faLA and faBASE. The patterns of DPA among the groups were reflected in the EPA pattern. The faEPA group had a 3 to 10 fold higher DPA compared to any of the other groups (Figure 11C). Similar to EPA, DPA was lowest in the lnLA, faLA and faBASE groups. DHA was the highest in the faDHA animals, being 8 fold higher compared to the other groups. DHA was not different among the other groups (Figure 11D).

In terms of n6 fatty acids, the lnLA group had the highest LA and faLA had the second highest LA, which was similar to faDHA and faBASE (Figure 12A). The lnLA group also had the highest γ-linolenic acid (GLA), followed by faLA, which was similar to faBASE (Figure 12B). The faEPA, faDHA and faALA groups had the least GLA, which was similar to faBASE. Dihommo-gamma-linolenic acid (DGLA), an intermediate in the conversion of GLA to AA was highest in lnLA while lowest in faALA, which was similar to faEPA and faBASE (Figure 12C). The faLA and faEPA groups had intermediate levels of GPA. The lnLA had the highest AA, followed by faLA and faBASE, while faALA, faEPA and faDHA had the least (Figure 12D).

Hepatic PL

There were no differences among the groups in relation to SFA or MUFA in the PL fraction (Table 15). The faLA group had the lowest proportion of PUFA in the PL fraction, but was similar to faALA and faEPA. The faDHA had the highest total n3

PUFA in the PL fraction, which was similar to the faEPA and faALA rats. The lnLA and faBASE had the highest total n6 while there were no differences seen among *fa/fa* experimental rats for n6 in the PL. The faBASE had the highest n6/n3 ratio followed by the lnLA and faLA, while the faALA, faEPA and faDHA had the lowest n6/n3 ratio.

Again, the n3 fatty acid profile of the diet generally reflected the fatty acid of the hepatic PL fraction. The faALA group had the highest ALA in the PL fraction and there were no differences among the other groups (Figure 13A). EPA was highest in the faEPA group, followed by faDHA and faALA, which were similar amongst each other (Figure 13B). The faBASE, faLA and lnLA had the lowest amount of EPA. The faEPA group had the highest amount of DPA followed by faALA (Figure 13C). The faBASE had intermediate amounts of DPA followed by faDHA while the lnLA and faLA groups had the lowest DPA. The faDHA group had the highest DHA, and faALA had the second highest DHA while the lnLA had the least (Figure 13D).

In relation to n6 fatty acids, the faDHA and lnLA had similar proportions of LA in the PL fraction (Figure 14A). The faLA had the lowest, which was similar to the faEPA. The lnLA group had the highest GLA and faEPA had the least, which was similar to faALA (Figure 12B). DGLA was highest in the faDHA and faALA groups and lowest in the lnLA group (Figure 12C). In relation to AA, lnLA, faLA and faBASE all had similar proportions, which were the highest amongst the groups. The faDHA group had the least AA, which was similar to faEPA.

Table 14: Hepatic TAG fatty acid composition

Totals	lnLA	faLA	faALA	faEPA	faDHA	faBASE
Total SFA[2] (g/100 g of lipids)	27.3 ± 2.5^{d}	40.7 ± 1.4^{ab}	42.1 ± 0.9^{ab}	39.1 ± 0.9^{bc}	31.9 ± 5.2^{cd}	46.4 ± 0.7^{a}
Total MUFA[2] (g/100 g of lipids)	18.4 ± 1.0^{d}	37.3 ± 0.8^{bc}	41.2 ± 0.6^{a}	34.5 ± 1.1^{c}	33.0 ± 2.4^{c}	39.4 ± 1.1^{ab}
Total PUFA[1] (g/100 g of lipids)	54.3 ± 3.3^{a}	20.6 ± 2.2^{d}	16.8 ± 0.9^{de}	26.4 ± 2.1^{c}	34.1 ± 3.1^{b}	14.1 ± 0.9^{e}
Total n3[2] (g/100 g of lipids)	3.64 ± 2.5^{c}	0.37 ± 0.1^{c}	8.77 ± 0.6^{b}	8.65 ± 0.8^{b}	19.5 ± 1.8^{a}	1.75 ± 0.2^{c}
Total n6[2] (g/100 g of lipids)	50.6 ± 5.7^{a}	20.3 ± 2.2^{b}	7.99 ± 0.3^{d}	17.7 ± 1.3^{bc}	14.6 ± 1.4^{bcd}	12.4 ± 0.8^{cd}
n6/n3 Ratio[2]	45.2 ± 11.4^{b}	64.2 ± 7.4^{a}	0.92 ± 0.03^{c}	2.09 ± 0.1^{c}	0.75 ± 0.05^{c}	7.20 ± 0.3^{c}

Values are expressed as means ± SEM, n =6. Different superscript letters indicate significant differences ($p<0.05$) among the means.
[1]Data were log transformed prior to ANOVA.
[2]Data were analyzed using non-parametric testing for statistical analysis.

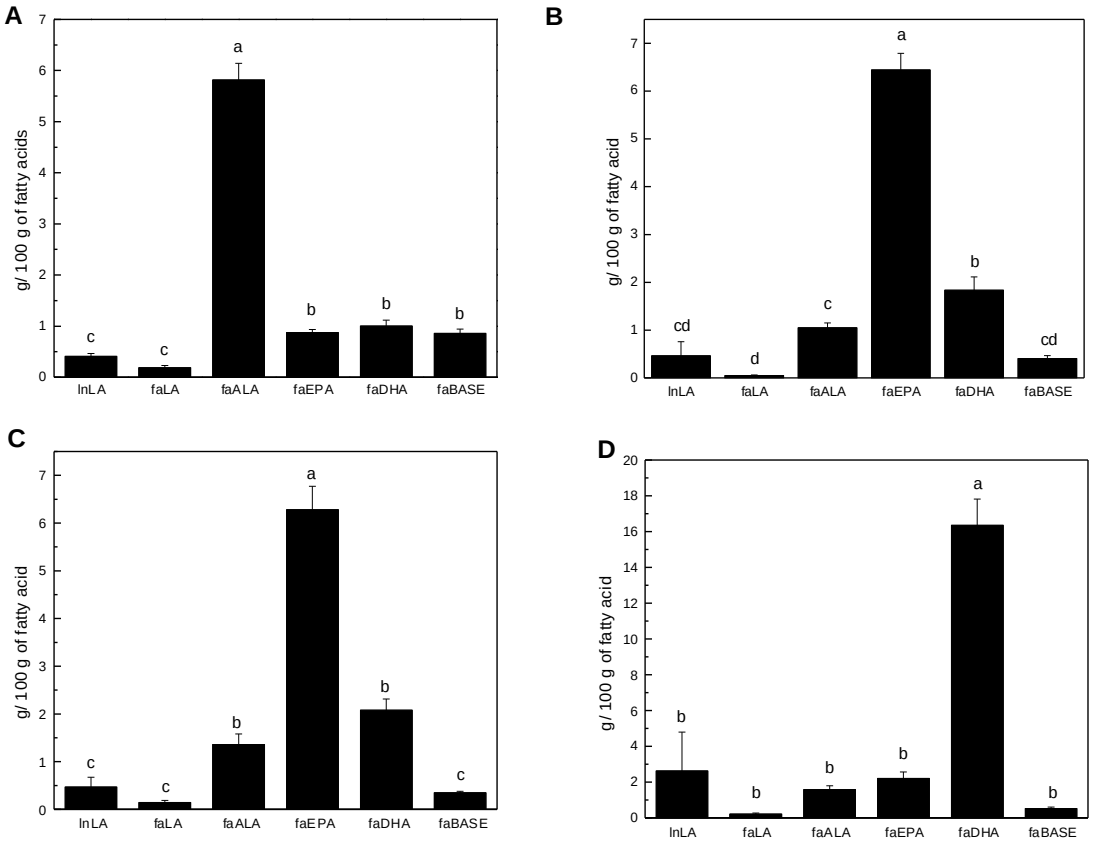


Figure 11: Hepatic TAG n3 fatty acids. ALA (C18:3n3) (A), EPA (C20:5n3) (B), DPA (C22:5n3) (C), and DHA (C22:6n3) (D) as g/100 g of fatty acid presented as means ± SEM, n=6. Different letters indicate significant differences ($p<0.05$) among the means. Data were analyzed by non-parametric statistics.

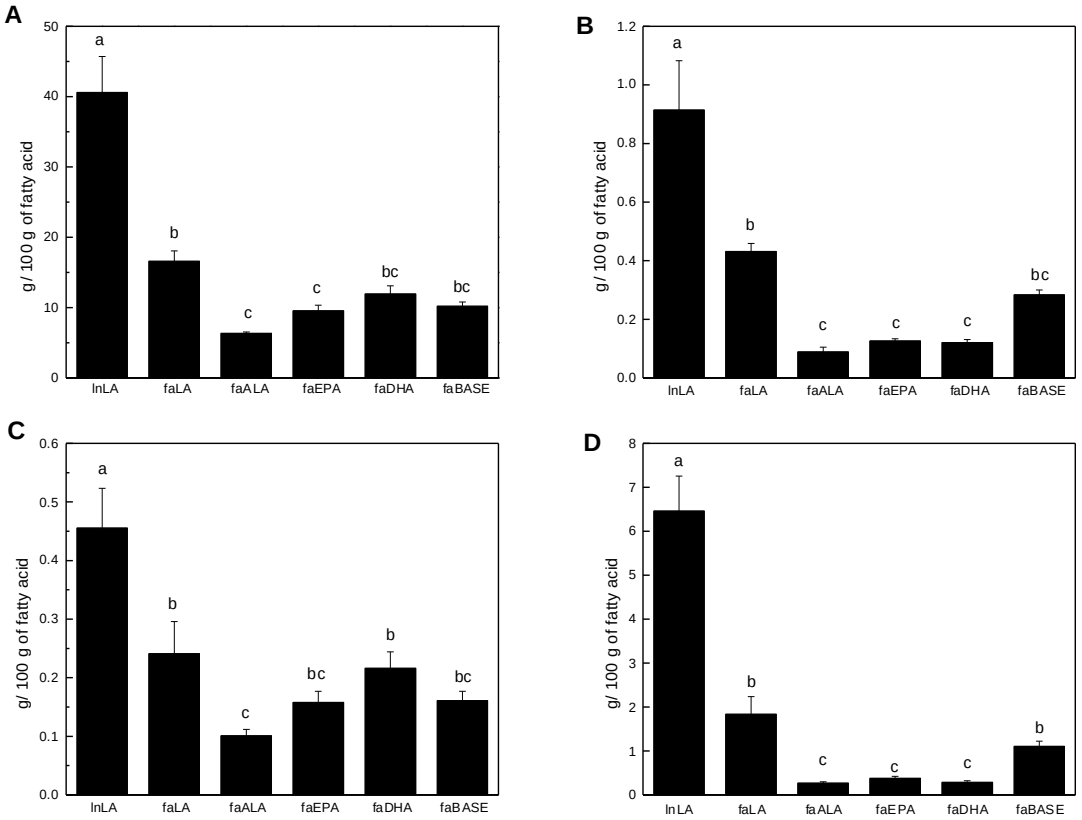


Figure 12: Hepatic TAG n6 fatty acids. LA (C18:2n6)(A), GLA (C18:3n6)(B), DGLA (C20:3n6)(C) and AA (C20:4n6)(D) as g/100 g of fatty acid presented as means ± SEM, n=6. Different letters indicate significant differences ($p<0.05$) among the means. Data were analyzed using non-parametric statistics except for (D) C20:4n6 where the data were log transformed prior to ANOVA.

Table 15: Hepatic PL fatty acid composition

Totals	lnLA	faLA	faALA	faEPA	faDHA	faBASE
Total SFA[1] (g/100 g of lipids)	43.9 ± 0.1	46.0 ± 0.5	45.3 ± 0.7	45.5 ± 0.6	44.4 ± 0.4	43.5 ± 0.1
Total MUFA (g/100 g of lipids)	5.89 ± 0.1	9.05 ± 2.5	6.62 ± 0.2	7.25 ± 0.2	6.07 ± 0.2	7.22 ± 1.9
Total PUFA (g/100 g of lipids)	50.1 ± 0.1^{a}	45.0 ± 2.6^{b}	48.0 ± 0.6^{ab}	47.2 ± 0.5^{ab}	50.0 ± 0.3^{a}	49.3 ± 1.6^{a}
Total n3[2] (g/100 g of lipids)	8.7 ± 0.4^{d}	12.8 ± 5^{bc}	17.4 ± 3.6^{ab}	15.2 ± 0.3^{abc}	21.5 ± 0.4^{a}	4.85 ± 0.4^{cd}
Total n6[2] (g/100 g of lipids)	41.4 ± 0.4^{a}	32.2 ± 5.6^{b}	30.6 ± 3.5^{b}	32.0 ± 0.2^{b}	28.0 ± 0.4^{b}	44.5 ± 1.4^{a}
n6/n3 Ratio[2]	4.81 ± 0.2^{b}	4.36 ± 1.1^{b}	2.11 ± 0.4^{c}	2.10 ± 0.03^{c}	1.30 ± 0.04^{c}	9.39 ± 0.6^{a}

Values are expressed as means ± SEM, n =6. Different superscript letters indicate significant differences ($p<0.05$) among the means and an absence of superscript letters indicates no significant differences.

[1]Data were log transformed prior to ANOVA.

[2]Data were analyzed using non-parametric testing for statistical analysis.

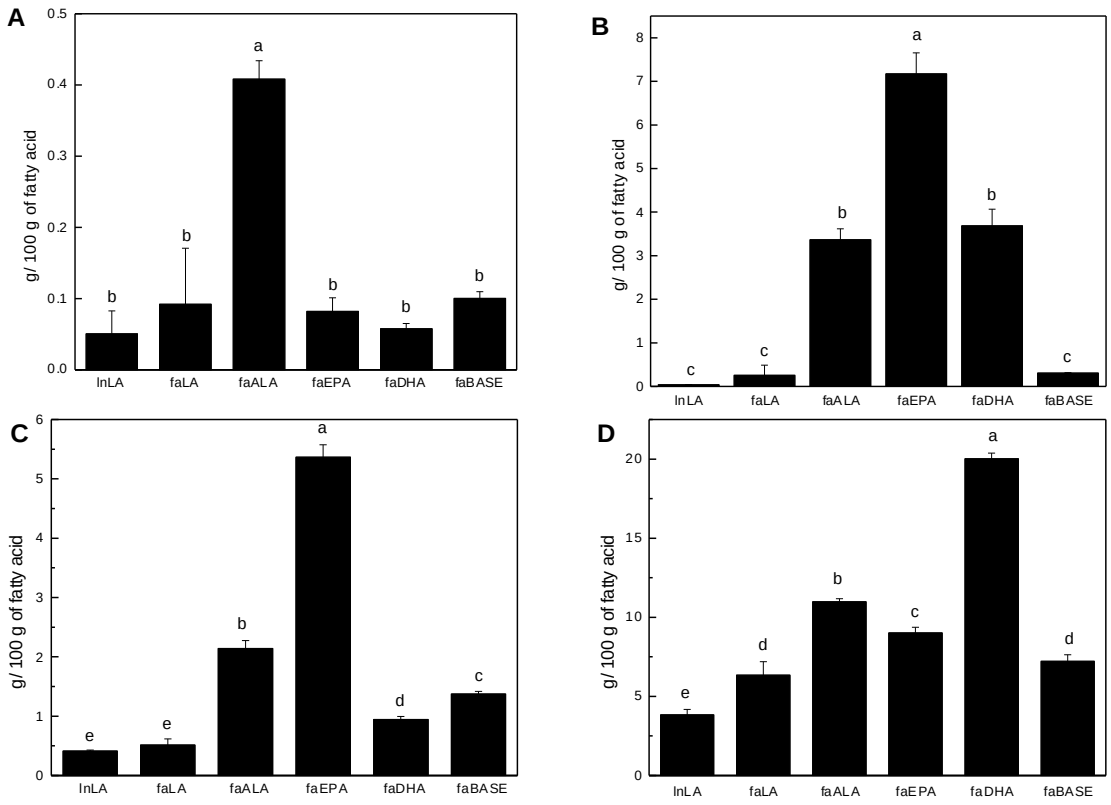


Figure 13: Hepatic PL n3 for fatty acids. ALA (C18:3n3) (A), EPA (C20:5n3) (B), DPA (C22:5n3) (C), and DHA (C22:6n3) (D) as g/100 g of fatty acid presented as means ± SEM, n=6. Different letters indicate significant differences ($p<0.05$) among the means. Data were analyzed by non-parametric statistics.

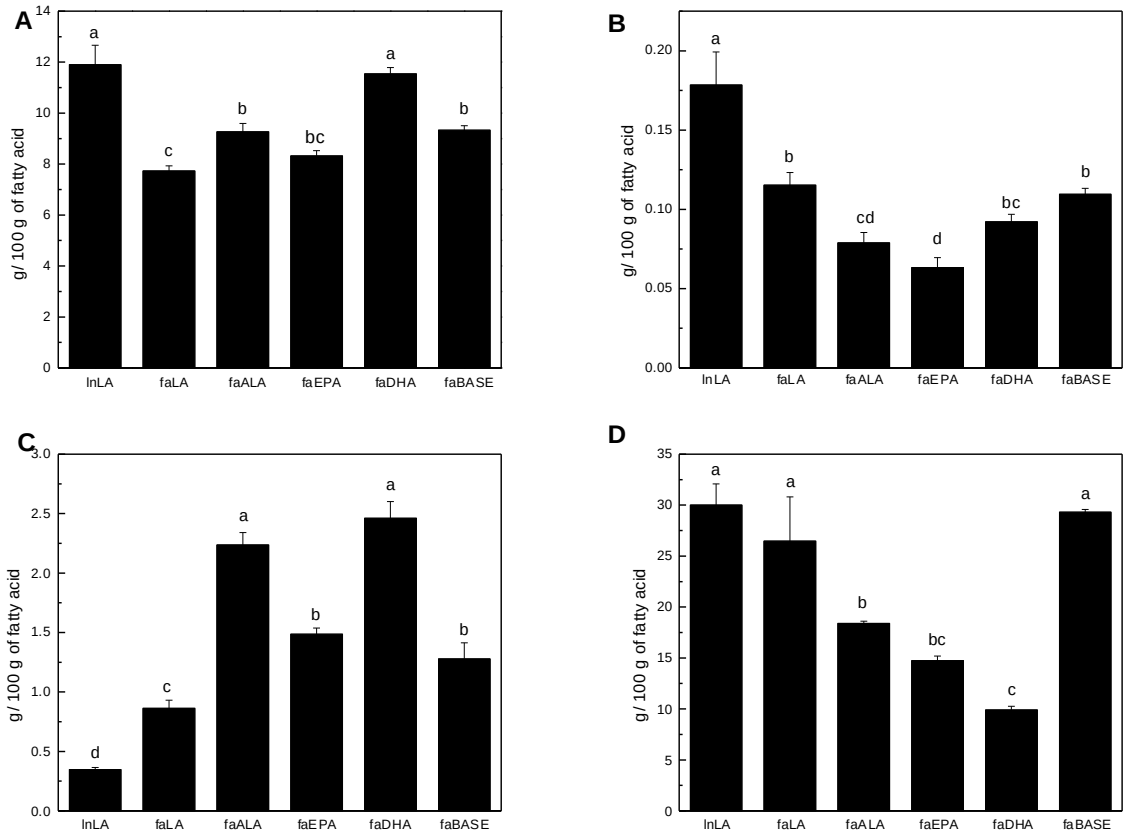


Figure 14: Hepatic PL n6 fatty acids. LA (C18:2n6)(A), GLA (C18:3n6)(B), DGLA (C20:3n6)(C), AA (C20:4n6)(D) as g/100 g of fatty acid presented as means ± SEM, n=6. Different superscript letters indicate significant differences ($p<0.05$) among the means. C18:3n6 was log transformed prior to ANOVA. C18:2n6 and C20:4n6 were analyzed using non-parametric statistics.

Serum biomarkers for hepatic function

Liver function was determined by measuring liver enzymes. ALT and AST are two enzymes that are commonly found in the liver, but when hepatic function is compromised levels in the serum rise (reviewed by Bulchandani and Harris, 2006). Parallel results were observed for ALT and AST in the serum (Figure 15A and B, respectively). The faALA group had the highest concentration and faBASE had the lowest of both enzymes. The other groups have intermediate levels of both enzymes with no differences among the faLA, faEPA, faDHA and lnLA groups.

Haptoglobin is an acute phase protein that can be used to determine inflammation (reviewed by Quaye, 2008). The lnLA and faDHA groups had the highest circulating haptoglobin, which were similar among each other. The faALA and faEPA had less circulating haptoglobin, which was similar to faBASE, but higher than faLA (Figure 15C).

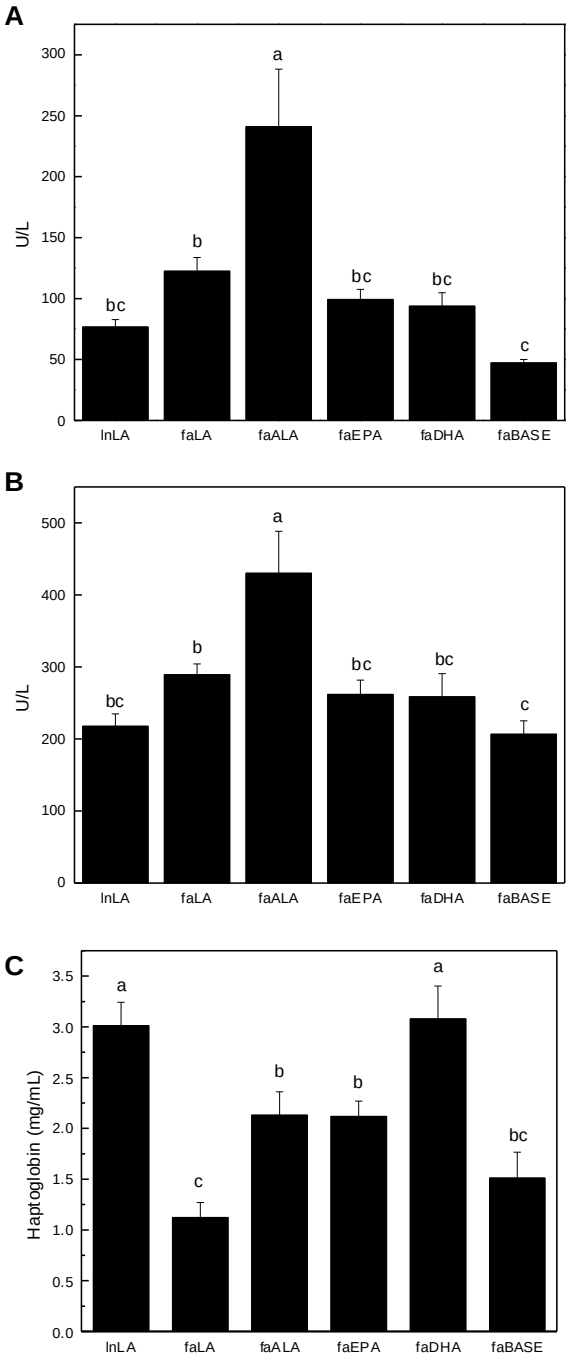


Figure 15: Serum AST (A), ALT (B) and haptoglobin (C). Values are expressed as means ± SEM, n =7-10. Different letters indicate significant differences ($p<0.05$) among the means.

Molecular markers of fatty acid oxidation, synthesis and uptake

SREBP-1c, pACC and ACC were used as molecular markers for fatty acid synthesis. SREBP-1c transcription factors are located in the bilayer of the endoplasmic reticulum as a helical hairpin (125 kDa), and must undergo 2 proteolytic cleavages to create an active cystolic fragment that enters the nucleus (68 kDa) (Brown et al., 2000). There were no differences seen in hepatic protein levels of SREBP-1c or activated SREBP-1c [68 kDa/(68 kDa + 125 kDa)], pACC and ACC. ACO, PPARα and pPPARα were used as molecular markers for fatty acid oxidation (Table 16 and Figure 16). There were no differences seen in hepatic protein levels of ACO. There were differences seen in pPPAR relative to β-tubulin. The lnLA and the faALA group had the highest relative level of pPPARα, which was similar to faLA. The faDHA group had the least, which was similar to faEPA and faBASE. Oddly, there were no differences in pPPARα relative to PPARα, although a similar trend as the pPPARα could be seen. This might have been due to the variability present in the blot limiting the ability to see significances. HIG2 was used as a marker for hepatic fatty uptake into the lipid droplets present in the liver. There were no significant differences seen in HIG2 among any of the groups.

Table 16: Molecular markers of hepatic oxidation, synthesis and uptake

	lnLA	faLA	faALA	faEPA	faDHA	faBASE
SREBP-1c (C-20) 125 kDa[1]	0.4 ± 0.1	0.6 ± 0.1	0.5 ± 0.1	0.5 ± 0.1	0.5 ± 0.1	0.7 ± 0.2
SREBP-1c (C-20) 68 kDa[1]	0.6 ± 0.1	0.9 ± 0.1	1.1 ± 0.3	0.7 ± 0.1	0.9 ± 0.2	1.2 ± 0.3
SREBP-1c (C-20) Activation[2]	0.59 ± 0.05	0.65 ± 0.05	0.62 ± 0.07	0.60 ± 0.05	0.63 ± 0.05	0.63 ± 0.02
ACC[3]	2.9 ±0.6	8.1 ± 1.8	5.6 ± 0.4	6.4 ± 2.1	3.6 ± 0.4	6.9 ± 2.6
pACC[3]	1.5 ± 0.2	1.0 ± 0.2	1.1 ± 0.2	0.9 ± 0.2	1.4 ± 0.2	1.3 ± 0.2
ACC Activation[4]	1.3 ± 0.4	1.0 ± 0.3	1.1 0.4	0.9 ± 0.3	1.4 ± 0.4	1.5 ± 0.4
ACOX1[1]	2.4 ±0.5	2.6 ± 0.9	3.1 ± 0.9	5.2 ± 1.6	3.1 ± 0.5	2.5 ± 0.4
PPARα[1]	2.2 ± 0.5	2.3 ± 0.5	2.5 ± 0.5	1.7 ± 0.6	1.5 ± 0.4	1.9 ± 0.4
pPPARα[1,5]	1.8 ± 0.2^{b}	2.9 ± 0.4^{ab}	3.4 ± 0.5^{a}	1.8 ± 0.3^{b}	1.5 ± 0.3^{b}	4.3 ± 0.8^{a}
PPARα Activation[6]	1.2 ± 0.4	1.4 ± 0.2	1.6 ± 0.3	1.7 ± 0.6	1.2 ± 0.2	2.4 ± 0.5
HIG2 (C-14)[1]	1.2 ± 0.1	1.0 ± 0.2	1.1 ± 0.3	1.3 ± 0.2	1.7 ± 0.3	1.3 ± 0.3

Values are expressed as means (arbitrary units) ± SEM, n =3-6. Different superscript letters indicate significant differences ($p<0.05$) among the means and an absence of superscript letters indicates no significant differences. All data were adjusted by the loading control (β-tubulin or EEF2) or the phosphorlayted form if available, to control for differences in protein loading

[1]Data relative to β-tubulin

[2]Activation state calculated as SREBP-1c (68 kDa)/[SREBP-1 (68 kDa) + SREBP-1 (125 kDa)]

[3]Data relative to EEF2

[4]Data calculated as pACC/ACC

[5]Data were log transformed prior to ANOVA

[6]Data calculated as pPPARα/PPARα

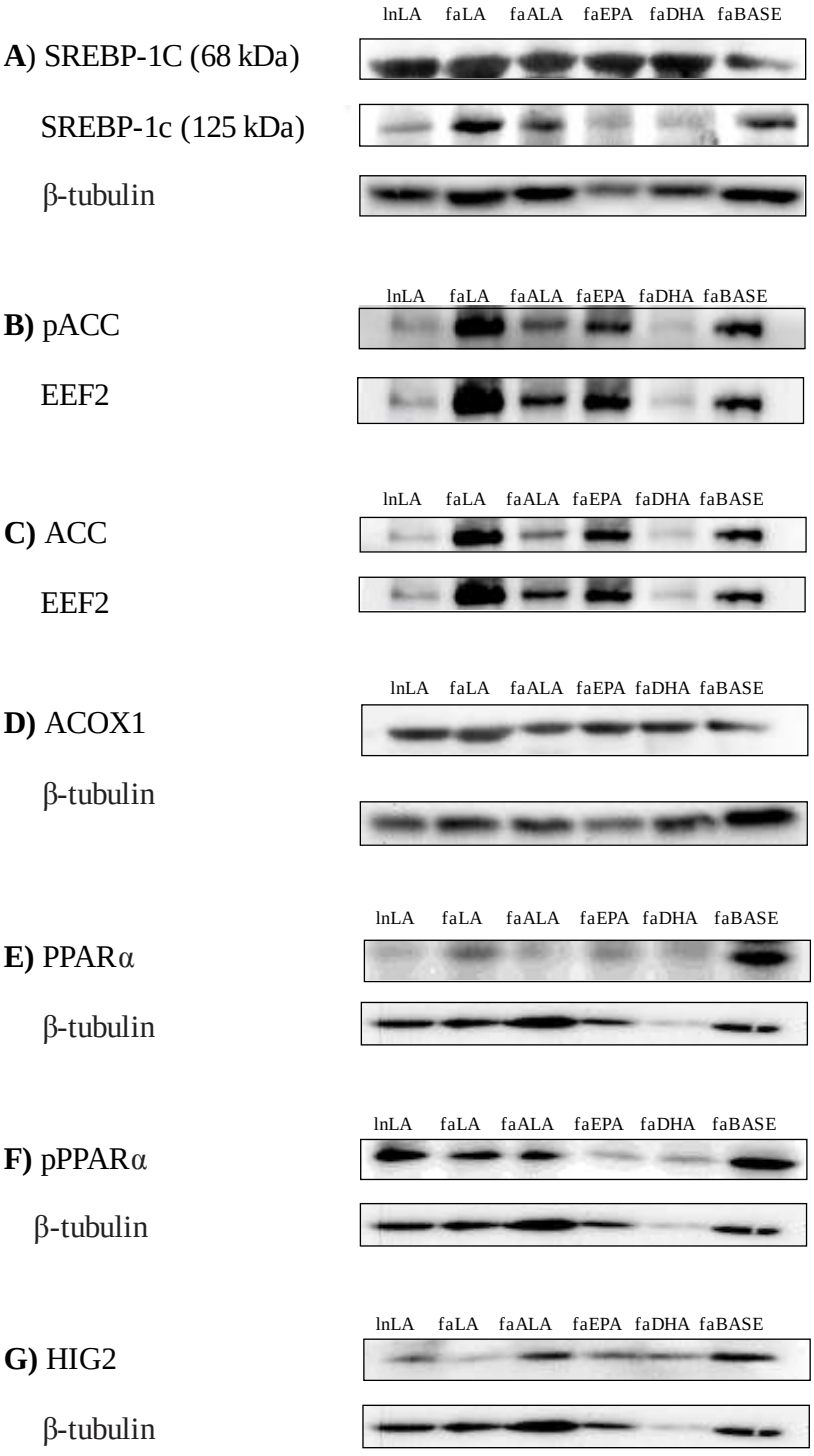


Figure 16: Representative Blots. Relative levels of SREBP-1c (A), pACC (B), ACC (C), ACOX1 (D), PPARα (E), pPPARα (F), HIG2 (G) were determined by Western blotting of liver tissue samples

Adipose function

Adipocyte size

There were no differences in the average size of adipocytes among any of the groups (Figure 17A). Chi-squared testing indicated that there were differences among the dietary groups for adipocytes <500 μm^2, 2500-3000 μm^2 range and >3000 μm^2 (Figure 17B). The faBASE group had 25% of its adipocytes in the smallest size range (<500 μm^2), compared to 17% for faEPA and faDHA, 10% for lnLA and faALA, and 6% for faLA. The faLA group had 20% of its adipocytes in the largest size range (>3000 μm^2), compared to 12% for faALA and 3-6% for the remaining groups.

Serum biomarkers for adipocyte dysfunction

Circulating adipokines were used as markers of adipocyte dysfunction. Serum leptin was elevated in all *fa/fa* groups except faEPA compared to lnLA (Figure 19A). Serum resistin and adiponectin were not different among the groups (Figure 19B and C, respectively). MCP-1 is an adipokine that is related to inflammation and adipose dysfunction (reviewed by Hui et al., 2013). The faEPA group had the lowest circulating MCP-1, which was similar to lnLA, faLA and faDHA. Figure 19D). There was also no detectable TNF-α in the termination serum of the experimental animals (data not shown).

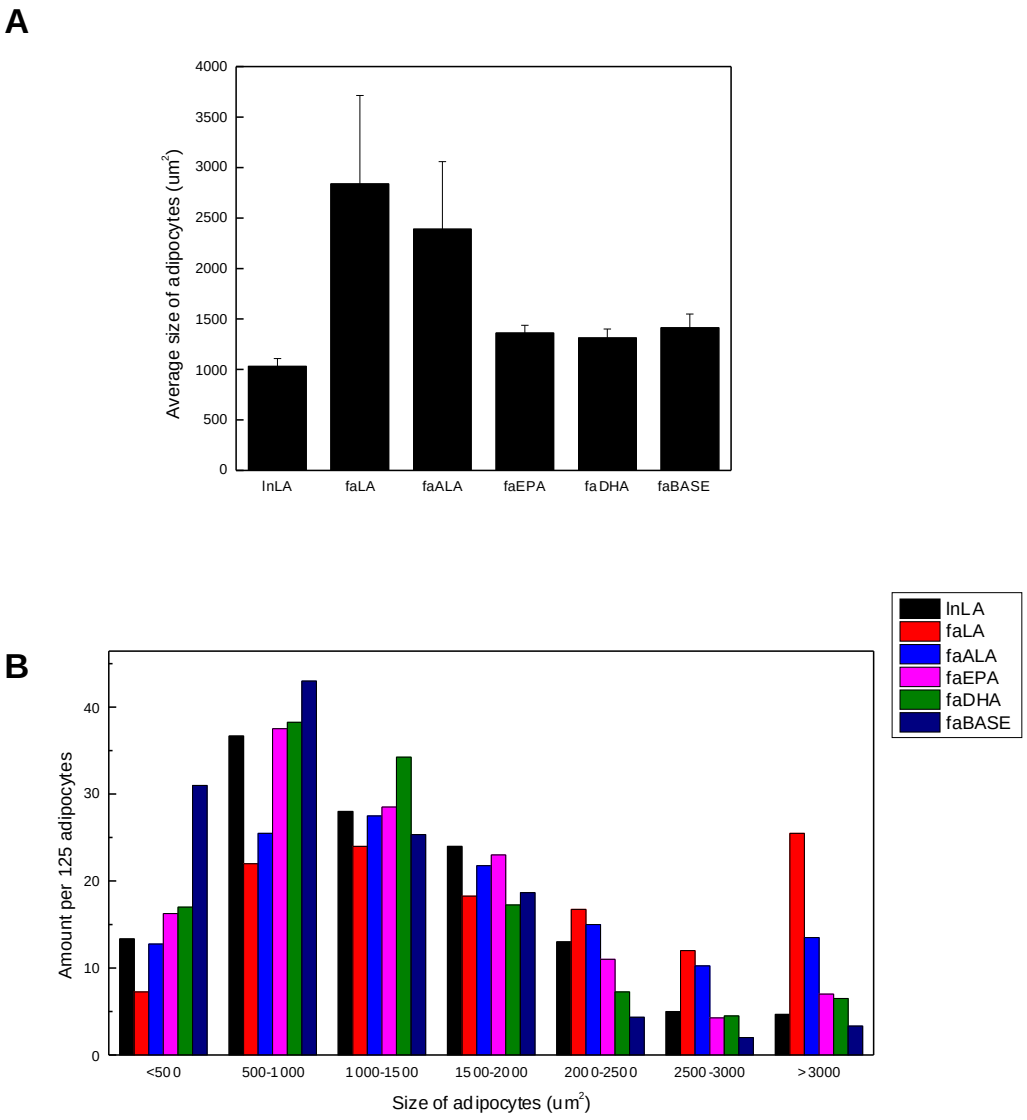


Figure 17: Average adipocyte size (A) and distribution of the sizes of adipocytes (B). (A) Values are expressed as means ± SEM, n =3-4. (B) Values are expressed as number per 100 droplets.

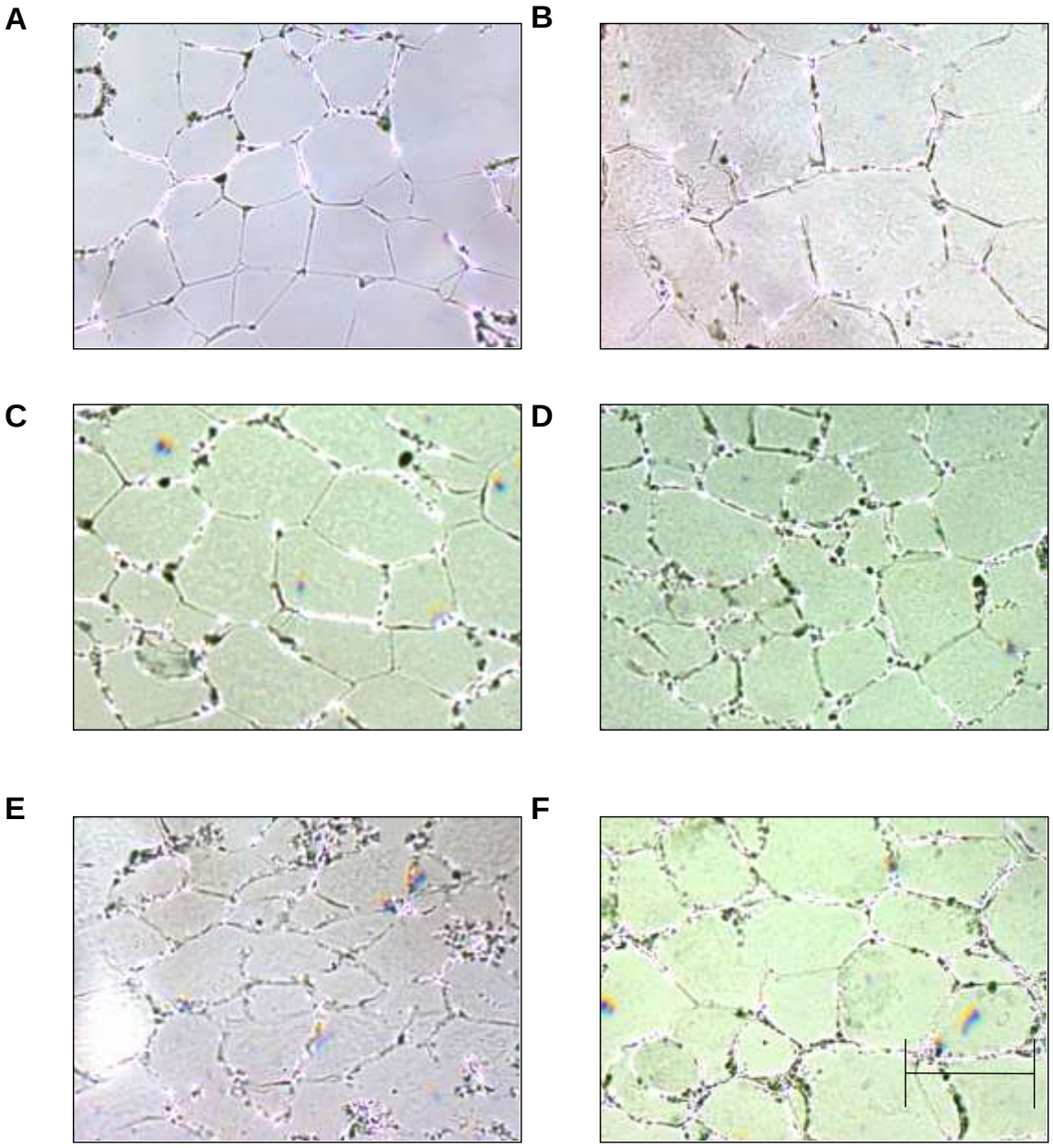


Figure 18: Representative images of adipose tissue. lnLA(A), faLA(B), faALA(C), faEPA(D), faDHA(E) and faBASE(F). Scale bar equal to 0.05 mm.

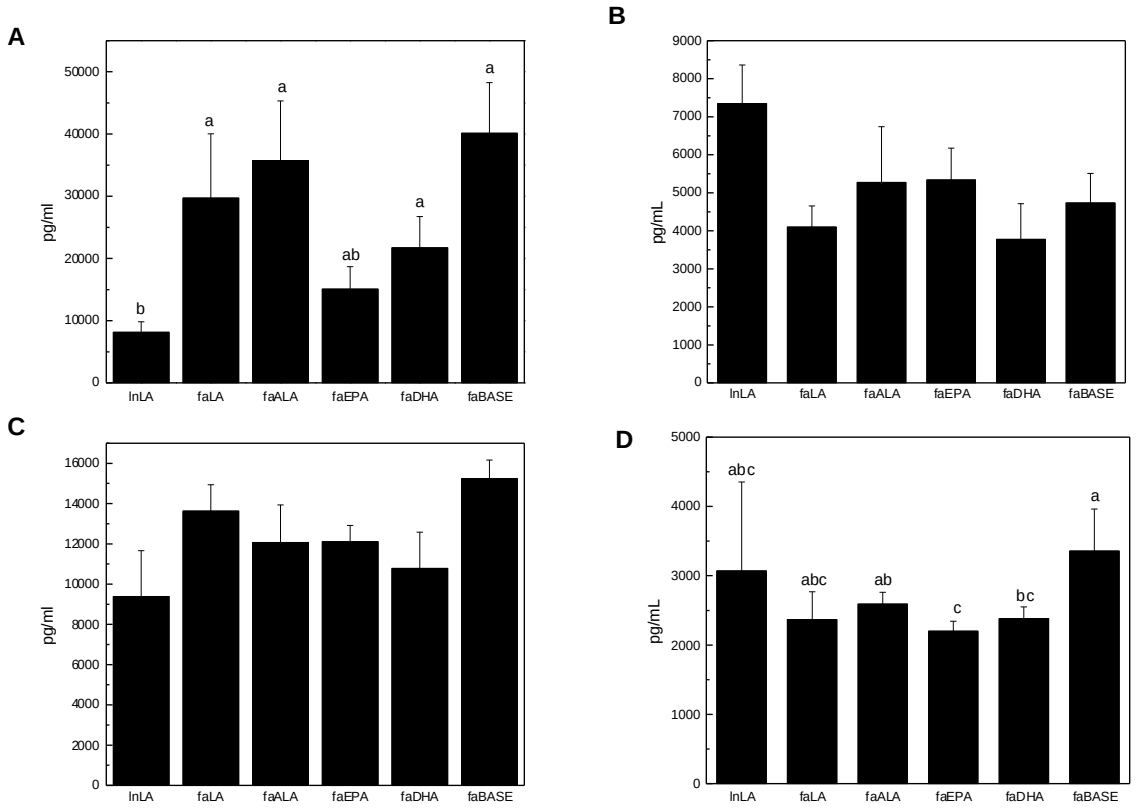


Figure 19: Serum leptin (A), resistin (B), adiponectin (C) and MCP-1 (D). Values are expressed as means ± SEM, n =5-10. Different letters indicate significant differences ($p<0.05$) among the means and an absence of letters indicates no significant differences. Leptin data were log transformed prior to ANOVA.

DISCUSSION

Plant n3 oil versus marine n3 oil on physical characteristics

At the end of the study, the plant oil fed groups (both faLA and faALA) had ~10% higher final body weight than the marine oil fed groups (faEPA and faDHA), corrected for body weight. These results were parallel to the total feed intake. All of the *fa/fa* experimental animals had similar total body fat (40-47% of body weight), while the lean Zucker rats had the least amount of body fat (12% of body weight). There were minor differences seen in the individual fat depots. The faLA control displayed a higher amount of subcutaneous fat corrected for body weight. Furthermore, the faLA group also more epididymal fat compared to the other *fa/fa* groups, but similar to faEPA. The plant n3 oil group had more mesenteric fat, corrected for body weight, compared to the group fed the DHA diet, but not the EPA diet. The DHA fed group had a similar percentage of visceral fat as the faBASE. There were no differences in peri-renal fat among the *fa/fa* experimental groups.

Zucker rats that express the *fa* allele are significantly hyperphagic compared to their lean littermates as early as 17 days of age, and continuing during the first 16 weeks of life (Stern and Johnson, 1977; Vasselli et al., 1980). Previous research on pharmacological and dietary manipulations has succeeded to varying extents at reducing the hyperphagia present in the *fa/fa* Zucker rats (Kava et al., 1990). Furthermore, studies that have assessed jejunoileal bypass surgery on *fa/fa* Zucker rats have been successful at decreasing feed intake and consequently body weight (Greenwood et al., 1982). However, studies have shown that such treatments do not normalize the obese body composition

(Cleary et al., 1980). Although feed restriction can decrease body weight, it does not affect overall body composition and the *fa/fa* Zucker rats maintain a body composition that is approximately 50% fat compared to less than 20% fat in their lean counterparts. In this present study, the marine oil fed rats showed reduced hyperphagia compared to the plant oil and control experimental groups which was reflected in their lower final body weight. The faEPA and faDHA groups consumed ~30% less feed than the faLA and faEPA for the first week, and they continued to consume ~10-15% less feed over the course of a week for the duration of the study. This might be explained by the palatability of the high percentage of fish oil present in the EPA and DHA diets. The rats might have taken longer to get accustomed to the feed versus the faLA and faALA groups. It has also been previously hypothesized that the supplementation of EPA and DHA can reduce the reward associated with food, thereby reducing appetite and food intake through the modulation of the endocannabinoid and mesocorticolimbic pathways (reviewed by Golub et al., 2011). A limitation of the present study is the lack of a pair weight group, as reduced feed intake and body weight can affect various parameters. Glucose tolerance can be improved by a reduction in body weight, but this was not the case for the faLA group as it displayed improvements in insulin resistance despite having one of the heaviest body weights. Interestingly, both faEPA and faDHA groups had lower body weights compared to the faLA and faALA groups, however, the faDHA had more improvements in glucose handling and insulin-related parameters than the faEPA group. This study also shows that there is a slight improvement of lipid accumulation in particular fat pads due to the DHA diet, but the overall body composition of the DHA fed

rats was nowhere near their lean counterparts. Furthermore, a reduction of total body fat was not shown.

Past studies have shown varying results in regard to n3 PUFA feeding and changes in body weight. Gillam et al. (2009) found that the addition of an oil from a flax source containing 3.5% (w/w) ALA or fish oil containing 0.8 % (w/w) EPA and 0.9% (w/w) DHA did not alter the total feed intake in *fa/fa* Zucker rats when dietary fat made up 10% (w/w) of the diet. Furthermore, there were no differences seen in final body weights or adipose weights between the groups. The amount of ALA in the diet in the Gillam et al. (2009) study was similar to the present study. Even though they did not test EPA and DHA exclusively, the fish oil used contained a 1:1 ratio and 0.8% EPA and 0.9% DHA (w/w), which was less than the amount of EPA or DHA added in the present study. Gillam et al. (2009) also used the same animal model; therefore, it is not unexpected that similar results were seen. Similar results were seen by Botelho et al. (2013) when comparing fish oil (EPA+DHA), algae oil (DHA) and echium oil (stearidonic acid (SDA)+ALA) supplements in LDL receptor deficient knockout mice. The mice were fed a high fat diet (34% [w/w] fat), and given an oral galvage of fish oil (0.44 g of EPA + 0.25 g of DHA), algae oil (0.77 g of DHA) or echium oil (2.8 g of SDA, which can be converted to EPA, + 6.7 g of ALA) daily. Lonzetti-Bargut et al. (2014) also found no differences in final body weights or adipose weights in LDL receptor-deficient knock-out mice fed low or high fat diets (4% or 27.8% [w/w] fat, respectively) with or without fish oil (0.9% or 5.95% [w/w] EPA + DHA).

In contrast, Samane et al. (2009) found that a high fat/high sucrose diet (39.6% [w/w] fat) containing 0.96% (w/w) EPA and 0.65% (w/w) DHA was able to prevent an

increase in adiposity and, although not significant (p=0.051), slightly reduced food intake compared to the rats given a high fat/high sucrose diet that did not contain any fish oil. Rokling-Anderson et al. (2009) also found that the supplementation of n3 PUFAs from a marine source (3.7% [w/w] EPA and 2.2% [w/w] DHA) decreased adiposity in Wistar rats compared to rats fed a lard-based control diet containing 21.5% (w/w) fat.

Plant n3 oil versus marine n3 oil on hepatic steatosis and function

There were no differences in liver weight or liver/body weight ratios among the *fa/fa* animals, but differences were seen in total liver lipids. Marine oil effectively prevented the progression of steatosis with the DHA and EPA fed groups having total liver lipid concentrations similar to the baseline animals. The EPA fed group was also more effective at preventing the progression of hepatic steatosis than the plant oil fed group, but not as effective as the DHA fed group. The faEPA had ~25% less liver lipid than the faALA while the faDHA had ~35% less than the faALA.

Hepatic lipid droplet assessment showed no differences in the average size of liver lipid droplets among the *fa/fa* experimental groups, but they were all significantly larger than the faBASE and lnLA groups, which were too small in size to quantify. Although no differences were seen in the average size of the droplets, there was a trend in the distribution of the size of the lipid droplets with faDHA having smaller droplets compared to the other groups and faLA having the largest. Previously, it was shown that the supplementation of DHA in the diet leads to a decrease in lipid droplet size in the liver. Le et al. (2012) found that a daily supplementation of 2% (w/w) of DHA daily via orogastric gavage had well preserved hepatic architecture with rare focal microvacuoles

in the cytoplasm of the mid zone hepatocytes of C57B16/J mice fed a high fat diet. They also found no evidence of macro or microvesicular steatosis in the liver sections with a daily supplementation of DHA delivered daily via orogastric gavage. In the past, it has been found that a daily oral gavage of fish oil containing 0.44 g of EPA + 0.25 g of DHA or echium oil containing 2.8 g of SDA and 6.7 g of ALA was able to attenuate hepatic steatosis whereas algae oil containing 0.77 g of DHA did not promote any protection against the hepatic steatosis induced by a high fat diet containing 34% (w/w) fat (Botelho et al., 2013). These results contrast with the findings obtained in the present study, as the ALA rich diet did not offer any protection against hepatic steatosis, while the DHA prevented the progression of hepatic steatosis in *fa/fa* Zucker rats.

The DHA group displayed the highest n3 in the TAG fraction, containing ~ 2.5× more n3 than faALA and faDHA, and ~4-6 times more than faLA, faBASE and lnLA. In relation to total n3 in the PL fraction, the faDHA group had the highest total n3, which was similar to the faALA and faEPA. The faDHA group also had the highest concentration of DHA in the both TAG (~5× more than faEPA and faALA) and PL (~2× more than faALA and 2.5× more than faEPA) fractions. Past studies have also found that n3 supplementation has led to increased levels of n3 PUFAs in the liver (Nuernberg et al., 2011; Botelho et al., 2013; Hanke et al., 2013). Nuemberg et al. (2011) found that when high body weight (DU6) mice were fed a high fat diet containing 3.3% (w/w) EPA and 4.3% (w/w) DHA, the progression of hepatic lipid accumulation was less when compared to a standard chow containing 7.2% fat (w/w).

Interestingly, the faEPA group had an increase EPA and DPA in the hepatic TAG fraction by 3-fold compared to the faDHA and 5-fold compared to the faALA, but

interestingly it did not increase the amount of DHA any differently than the faALA group. It has been shown in an endothelial cell model that EPA was predominantly elongated to DPA in the PL fraction when cells were treated with 5 µg/ml of EPA for 2 days, with little conversion to DHA (Kanayasu-Toyoda et al, 1996). In this present study the ALA fed group had a 2-fold increase in DPA in the PL fraction compared to the DHA fed group. These results were similar to Gillam et al. (2009), where they saw an increase in DPA in muscle and adipose TAG and PL fraction in *fa/fa* Zucker rats fed a diet containing 3% (w/w) ALA compared to the group fed a fish oil containing 0.8 % (w/w) EPA and 0.9% (w/w) DHA.

In the present study, the fatty acid composition of the liver showed that the n3 fed groups, regardless of body composition, had higher concentrations of n3 PUFAs in their liver, with the most abundant n3 PUFA being DHA in the faALA and faDHA groups and EPA in the faEPA PL fraction. The n3 oil present in the diet was shown to be most prevalent in the TAG fraction. Botelho et al. (2013) fed LDL receptor-deficient mice a diet containing 34% fat (w/w) along with a daily oral supplement of fish oil (0.44 g of EPA+ 0.25 g of DHA), echium oil (2.8 g of SDA and 6.7 g of ALA) or algae oil (0.77 g of DHA). They found that the echium oil supplemented group had the highest amount of EPA in the liver homogenate, which was similar to the group given the fish oil supplement. The echium oil fed group also had the lowest n6/n3 ratio in the liver homogenate. As previously mentioned, the echium oil and the fish oil supplementation were able to prevent the progression of hepatic steatosis more effectively than the algae oil supplement. It should also be noted that there was an increase of EPA and a decrease in the n6/n3 ratio in the liver homogenate, which may be might be related to the

prevention of hepatic steatosis. However, these results contrast with what was found in the present study as there were no differences seen in the *fa/fa* groups on the n3 PUFA rich diets in relation to n6/n3 ratio in hepatic TAGs or PLs, but the faDHA group showed the lowest degree of hepatic steatosis.

When Hanke et al. (2013) compared the consumption of high fat diets (31% w/w) with varying levels of ALA in OP-CD rats, the group fed the canola/flaxseed (C/F) oil blend (3:1 canola to flaxseed oil ratio and 6.1% ALA [w/w]) had less liver lipid compared the other groups fed diets containing <1% n3, indicating a delayed onset in the progression of hepatic steatosis. They also found that the rats fed the canola/flaxseed oil diet had the highest proportion of n3 in the hepatic phospholipid fraction compared to the other experimental groups. In regard to composition, the C/F fed group had the highest ALA in the hepatic TAG fraction, but lower EPA than the soybean oil fed group. There were no differences among the groups in relation to DHA. In regards to n3 PUFAs in the PL fraction, there were no differences among the groups in relation to ALA, but the C/F group had a 9.4-82.8 fold higher EPA level than the other groups and the highest amount of DHA present.

A proposed mechanism to explain these findings is that dietary n3 fatty acids regulate hepatic gene expression by targeting two major transcriptional regulatory networks, PPARα and SREBP-1c. Through the expression, or lack of expression, of these proteins, they are able to control glycolysis, *de novo* lipogenesis and fatty acid elongation, desaturation and oxidation (reviewed by Di Minno et al., 2012). When examining molecular markers for hepatic fatty acid oxidation, there were no changes seen in the activation state of PPARα; this might have been due to the variability present in the blot

limiting the ability to detect significant changes. There were differences seen in the levels of pPPARα compared to the loading control (β-tubulin), with the plant n3 oil being more effective than the groups fed marine oils at increasing pPPARα. There were also no differences seen in ACO or SREBP-1c levels or their activation state.

Although past studies have shown contrasting results connecting PPARα and ALA, in this study we did see that n3 plant oil was more effective at increasing hepatic PPARα expression than the marine oils in an obese model. Similarly, Zhang et al. (2014) compared Sprage Dawley rats fed either a control diet containing 4.3% fat (w/w) or high fat diet (23.5% [w/w] fat) with 15.7% (w/w) ALA from perilla oil for 16 weeks. They found that the high fat ALA supplemented group showed a 2.5 fold increase in mRNA expression of PPARα, and a 2 fold increase in mRNA expression of SREBP-1, but no differences were seen in protein level for either marker. Chechi et al. (2010) saw an increase in hepatic PPARα mRNA level only in the obese SHR/NDmcr-cp rats and not their lean counterpart even though the obese and lean rats were fed the same diet containing 5% ALA (w/w).

In contrast, Hanke et al. (2013) found no differences in PPARα, ACO or SREBP-1 expression among OP-CD rats fed high fat diets (31% [w/w]) with varying levels of ALA. Gillam et al. (2009) also found no difference in liver mRNA levels of PPARα or ACO in *fa/fa* Zucker rats fed a high ALA diet (3.5% [w/w]), or a fish oil diet (0.8% EPA [w/w]+0.9% DHA [w/w]). However, Lonzetti-Bargut et al. (2014) found that a high fish oil diet containing 5.96% (w/w) EPA+DHA, increased SREBP-1 expression, and decreased PPARα compared to a lower fat fish oil diet containing 0.9% (w/w)

EPA+DHA and a standard chow diet. These results contradict the proposed benefits of n3 PUFA supplementation in regards to PPARα and SREBP-1 expression.

There were no differences in ACC or pACC, which were used as downstream indicators of fatty acid synthesis. Hanke et al. (2013) also found no differences in hepatic ACC expression in OP-CD rats fed high fat diets (31% [w/w]) with varying levels of ALA. In contrast, Zhang et al. (2014) found that a high fat diet (23.5% [w/w]) containing 15.7% (w/w) ALA was able to decrease ACC protein expression by 2 fold compared to a group fed a control diet containing 4.3% fat (w/w). Rokling-Anderson et al (2009) found that a high fat diet containing of fish oil increased hepatic ACC mRNA levels compared to a high fat diet containing lard. Although not explored by this study, other proposed theories include the reduction of TAG-synthesizing enzymes, such as diacylgylerol acyltransferase (DGAT) or phosphatidic acid phosphohydrolase and an increases in PL synthesis, which could drain diacylglycerol away from DGAT (reviewed by Harris and Bulchandani, 2013).

When examining serum biomarkers for hepatic function, n3 marine oil did a more effective job at maintaining healthy levels of ALT and AST than plant n3 oil. The n3 plant oil fed group displayed the highest level of both biomarkers, while the baseline group had the lowest. There were no further differences seen among the other groups. Similar results were found in previous literature. Hanke et al. (2013) found that the supplementation of conventional canola oil, rich in MUFAs and ALA, increased both circulating ALT and AST compared to groups fed diets rich in SFAs or LA in diet-induced obese rats. Furthermore, Valenzuela et al (2013) showed that daily oral supplementation of a fish oil mixture (EPA+DHA) did not change circulating ALT or

AST levels in C57BL/6J mice. These results are contradictory to Poudyal et al. (2012), who showed that marine oil (both EPA and DHA), but not n3 plant oil supplementation, to a regular fat diet increases both serum ALT and AST in Wistar rats. Furthermore a further addition of plant or marine based n3 PUFAs at 3% (w/w) all decreased circulating AST in Wistar rats already being fed a high fat diet containing 24% fat (w/w).

In this present study, the faDHA group had the highest circulating level of haptoglobin, which was similar to lnLA, while the faLA had the lowest value. The faALA and faEPA had intermediate levels. These results are contradictory to Baranowski et al. (2012), who found that the supplementation of a high flax oil diet (2.8% [w/w] ALA) actually increased circulating haptoglobin levels in *fa/fa* Zucker rats when compared to a control diet containing <1% n3 PUFAs. ALT and AST are used as an indicator of hepatic function, while haptoglobin is used as an indicator of chronic inflammation. Haptoglobin is a non-specific biomarker for systemic inflammation and this is a limitation. The faALA group had the most compromised hepatic function reflected by the increased level of both enzymes. While the faDHA group had lower levels of circulating ALT and AST, it also had the highest level of haptoglobin, showing that while hepatic function was not highly compromised, there were higher levels of chronic inflammation present.

Plant n3 oil versus marine n3 oil on MetS parameters and adipose function

Obesity and adiposity, two parameters used to assess MetS and adipose function, are described above.

Although no major differences were seen in regard to fasting serum glucose among the *fa/fa* experimental groups, some differences were seen in OGTT at specific time points. While no differences were seen when measuring the AUC for glucose each dietary group peaked at different time points. The lnLA group peaked at t15 with a blood glucose value of ~11 mmol/L and the faALA peaked at t30 with a similar blood glucose value. The faEPA and faDHA peaked at t60, with a value at ~10.3 mmol/L, which was still higher than faALA at that time point. By t120, blood glucose normalized to fasting levels in the faBASE, lnLA and faALA, while the faDHA, faEPA and faALA remained elevated. While values rose during an OGTT, the *fa/fa* rats are not considered to be hyperglycemia according to the WHO definition of >11.1 mmol/L for blood glucose 2 hours after a glucose load. When Gillam et al. (2009) conducted an OGTT in *fa/fa* Zucker rats fed a high ALA diet (3.5% w/w), or a fish oil diet (0.8% EPA [w/w] +0.9% DHA [w/w]), no differences were seen. Furthermore, Poudyal et al. (2012) found that the dietary supplementation of chia seed oil (4.2% [w/w] ALA improved glucose tolerance during an OGTT, determined by $AUC_{glucose}$, in Wistar rats on a high fat diet which contained 27% fat (w/w). There were no differences seen among the groups supplemented with the marine n3s, or the groups fed a low fat diet containing 0.8% (w/w) fat. Similarly, Lonzetti-Bargut et al. (2014) found that supplementation of fish oil (5.95% [w/w] EPA + DHA) was only effective at improving glucose values during an OGTT, determined by $AUC_{glucose}$, after a long term consumption of a high fat diet (27.8% [w/w]).

Fasting serum insulin among the *fa/fa* groups was lowest in the DHA fed group which was similar to the LA control. While the faLA and faDHA groups had statistically

similar serum insulin during OGTT, serum insulin spiked slightly in the faLA group at t15 and t30, and dropped onwards. In contrast, the faDHA group had higher serum insulin at t120 and but similar to the other groups. The EPA and ALA fed diets had higher fasting insulin and serum insulin during OGTT compared to the DHA and LA groups. During OGTT, the faEPA and faALA groups started out with similar values, but the faEPA group continued to fall and by t30, it had similar values as faDHA and faLA, while not being different than faALA. The plant n3 oil fed group had the highest value for $AUC_{insulin}$, which was similar to the EPA group, while the DHA fed group was 33% lower. There was no difference in $AUC_{insulin}$ between the EPA and DHA groups. The HOMA-IR scores were also elevated in the ALA and EPA oil fed groups compared to the lean control, but not the faDHA group. Although EPA was more effective at promoting lower fasting serum insulin than ALA, the DHA oil was shown to be most effective throughout.

Samane et al. (2013) found that insulinemia was present in Wistar rats consuming a diet containing 39.6% (w/w) fat at all time points except at baseline during an intraperitoneal glucose test (IPGTT) compared to a chow fed control with 5.6% fat (w/w). When a portion of the fat was replace with fish oil, which contained 0.96% (w/w) EPA and 0.65% (w/w) DHA it was able to reduced insulin at all time points making the insulin concentration similar to that of the group fed the standard chow control. The $AUC_{insulin}$ values for the fish oil group were 3 fold lower than the high-fat/high sucrose group, while being similar to the standard chow control. These results show that the incorporation of fish oil into the diet is able to reduce insulinemia onset by a high-fat/high sucrose diet. Based on the results from this present study, DHA is more effective than EPA at reducing

serum insulin. Therefore the findings in the study conducted by Samane et al (2013) could be primarily due to the DHA present in the fish oil, and not the EPA.

Gillam et al. (2009) found the *fa/fa* Zucker rats had ~21 fold higher serum insulin levels than the lean control fed safflower oil at all time points regardless of diet, which mimic the results seen in this present study for the genotype differences. Furthermore, they found that the *fa/fa* group fed safflower oil had lower serum insulin at t=60 compared to the *fa/fa* rats fed a diet rich in flax oil or fish oil, but there was no overall difference seen in HOMA-IR. The results pertaining to the effectiveness of safflower oil at reducing insulinemia were also seen in this present study. The LA control was quite beneficial at promoting insulin sensitivity based on HOMA-IR, more so than the EPA and ALA based diets but similar to DHA. In support of these observations, a recent publication has shown that the development of insulin resistance in *fa/fa* Zucker rats can be prevented by supplementation with LA. Matravadia et al. (2014) compared 3 diets in lean and *fa/fa* Zucker rats. The rats were fed diet containing 7% (w/w) fat from soybean oil, or diets containing 12% (w/w) fat with either a soybean oil/flax seed oil mix or a soybean oil/safflower oil mix. The soybean oil/flaxseed oil blend contained 3.2% (w/w) ALA, while the soybean oil/safflower oil blend contained 7.3% (w/w) LA, which were similar to amounts in the ALA and LA diets in the present study. They found that there was a 2 fold increase in insulinemia quantified via $AUC_{insulin}$ in the *fa/fa* Zucker rats fed the diet containing only soybean oil compared to their lean counterparts. This increase in serum insulin was was not seen in the groups with the added LA or ALA when comparing the two genotypes.

When measuring FGF-21 and PTP1B as hepatic markers of impaired glucose tolerance and insulin resistance, there were no changes in hepatic PTP1B protein levels with the experimental diets and there was no detectable FGF-21 by Western blotting of liver. There are currently no studies that assess PTP1B and improvements via a functional food and this is the first study to examine the effects of n3 PUFAs on PTP1B.

In regards to FGF-21, Villaroya et al. (2014) found that expression of genes encoding FGF-21, its receptors, and FGF-21 targets was unaltered by short-term n3 PUFA treatment. In the first part of their study, they fed 8 week old C57BL/6J mice a high fat diet containing 35% corn oil (w/w) or a high fat diet with n3 PUFAs (7.1% DHA [w/w] and 2.2% EPA [w/w]) or a control low fat diet containing 3% corn oil (w/w) for 5 weeks. They found that there was an increase in plasma FGF-21 in the n3 supplemented group compared to the non-supplemented high fat group and low fat control with no differences in mRNA levels of FGF-21. In the second half of the study, they extended the treatment period for an additional 8 weeks on the same mice, but this time they decreased the n3 PUFAs content in the diet to 2.4% DHA (w/w) + 0.7 % EPA (w/w). They found that there was still an increase in plasma FGF-21 in the n3 supplemented group compared to the non-supplemented high fat and low fat control groups with no further differences seen in hepatic FGF-21 mRNA levels. They concluded that FGF-21 levels do not appear to be a major mechanism through which n3 PUFAs ameliorate high fat diet associated metabolic disorders.

The plant n3 oil fed group had the highest total cholesterol, which was similar to the faLA, which were 2 fold higher than the other experimental groups. Among the n3 PUFA fed groups, LDL-C and HDL-C was highest in the faALA. The DHA fed group

had the lowest HDL-C among the *fa/fa* experimental groups. The ALA fed group had elevated serum TAG, compared to faEPA and faDHA but not faLA. A limitation of the present study is that rats are not the best representative model for lipid metabolism. Furthermore, unlike humans, rats carry a large portion of their cholesterol in the HDL-C fraction, versus humans where cholesterol is mainly carried by the LDL-C fractions. In humans, it has been previously suggested that marine sources of n3 PUFAs work as a ligand to increase HDL-C through stimulation of lipoprotein activity (reviewed by Poudyal et al., 2011). These results would contradict the results seen in the *fa/fa* Zucker rat model in this present study as the faDHA group exhibited the lowest HDL-C among the *fa/fa* groups; in humans this would be attributed to a negative outcome of the DHA supplementation, as we aim to increase HDL-C to decrease the risk of MetS.

Valenzuela et al. (2013) found that daily oral supplementation of EPA and DHA was able to reduce serum TAG in C57BL/6J mice fed either a control diet containing 4.3% fat (w/w) or 34.9% fat (w/w) compared to their unsupplemented diet matched counterparts. Interestingly, they found no differences in serum total cholesterol between the diet matched groups. In contrast, Gillam et al (2009) found no differences in fasting serum TAG in *fa/fa* Zucker rats fed a diet rich in flaxseed oil, fish oil or safflower oil, although they did find that there was a decreased in circulating FFA in the fish oil fed group. Although not reflected in this study, Zhang et al. (2014) has shown the potential for ALA to aid in the reduction of serum lipids when they compared Sprage Dawley rats fed either a control diet containing 4.3% (w/w) fat to a high fat diet (23.5% [w/w]) which contained 15.7% (w/w) ALA for 16 weeks. They found that the high fat perilla oil fed group had lower levels of serum TAG, total cholesterol and HDL-C. They found no

differences between the groups in relation to LDL-C. Past research has also suggested that 3-4 grams of n3 PUFAs regardless of source reduce TAG levels in human by decreasing TAG secretion from the liver (reviewed by Harris and Bulchandani, 2013). Although the faDHA had less hepatic steatosis than the other n3 PUFA fed groups and the faLA, the faDHA had higher serum TAGs than the faEPA. This may be due in part to the *fa/fa* rat model used in the present study.

The DHA fed group, while similar to the EPA fed group, had lower diastolic blood pressure compared to ALA fed group. Systolic blood pressure and MAP were not different amoung the experimental *fa/fa* rats. Previous research has shown that n3 PUFAs are able to reduce systolic blood pressure in humans, regardless of the source (reviewed by Poudyal et al., 2011). This could occur through mechanisms which include the inhibition of renin secretion, the reduction of thromboxane A_2 formation, the inhibition of angiotension converting enzyme (ACE), structural modification of the blood vessel and the inhibition of the Na^+/K^+-ATPasae and Na^+/Ca^{2+} exchange (Codde et al., 1984; Juan et al., 1986; Juan et al., 1987). Bond et al. (1989) did a study investigating the effects of diets containing fish oil or pectin on blood pressure in deoxycorticosterone acetate (DOCA)-salt hypertensive rats. They had three groups, one fed 8% (w/w) fish oil with 2% (w/w) safflower oil, one fed 10% (w/w) safflower oil with 5% (w/w) of pectin and one fed 10% safflower oil with 5% α-cellulose that served as the control. The other diets contained 6% NaCl with all rats receiving DOCA at 30 mg/kg body weight weekly. They found that the systolic blood pressure of the rats fed the fish oil was lower than that of the rats fed the control diet, while no differences were seen between the control and pectin group. This study showed that fish oil consumption was able to decrease systolic blood

pressure more effectively than an n6 rich diet, but unfortunately this study did not record changes in diastolic pressure, which would relate to the changes seen in this present study. Hanke et al. (2012) reported both systolic and diastolic blood pressure and found that there were no differences in either when comparing varying levels of ALA ranging from 0-6.1% (w/w) in OP-CD rats fed high fat diets.

This present study showed no differences in the average size of adipocytes, although the faLA had noticeable more large adipocytes (>3000 μm^2) than the rest of the groups. Both the faLA and faALA groups also had more adipocytes in the 2500-3000 μm^2 range than the marine oil groups. The results also showed a high variability in the SEM of the mean particularly for the faLA and faALA groups, and a reduction of the SEM would likely show differences among the groups. A possible way to reduce the SEM would be to increase the sample size, as the sample size used for the statistical analysis was only 3-4 animals. Another way woud be to take more measurements per animal, as only 125 measurements were taken. Although not seen in this study, Baranowski et al. (2012) found that there was a 2 fold difference in adipocyte size in *fa/fa* Zucker rats fed a flax rich diet (2.8% [w/w] ALA) versus *fa/fa* Zucker rats fed a safflower control, indicating the potential for n3 PUFAs to minimize lipid deposition in adipocytes.

Adipokines reflect adipose function as an increase in adipocyte cell size leads to adipose cell dysfunction in obesity due to an imbalance of pro- and anti-inflammatory factors produced by adipocytes (Fernandez-Riejos et al., 2010). Adipose tissue from obese individuals has been shown to release TNF-α which stimulates adipocytes to secrete MCP-1, leading to macrophage recruitment (reviewed by Hui et al., 2013). Furthermore, it has been suggested that T-cells are among the first immune cells to enter

the adipose tissue and may stimulate pre-adipocytes to further recruit macrophages through further secretion of MCP-1 (Kintscher et al, 2008).

In this present study, we found no detectable amounts of TNF-α in the serum and the lnLA group had the lowest circulating MCP-1 compared to all the other *fa/fa* groups except faEPA. While the present study found no detectable serum TNF-α, Lionetti et al. (2014) found that TNF-α and MCP-1 were elevated in male Wistar rats fed a high fat diet containing 21.8% (w/w) of lard compared to a standard chow control. In the same study, rats fed a diet containing 21.8% (w/w) of fish oil was able to reduce the circulating concentrations of TNF-α and MCP-1 compared to the diet containing 21.8% (w/w) lard, but it was unable to reduce them to levels similar to the standard chow control. There was a decrease in serum leptin seen in the EPA fed animals but not any other n3 PUFA fed animals. Serum leptin would be expected to be decreased in the faDHA group given their reduced body mass and smaller adipocyte size, however, this was not the case. Past studies have not shown major significant changes in serum leptin related to n3 PUFA supplementation from a marine or a plant source but there were differences due to genotype (Baranowski et al., 2012; Gillam et al., 2009; Rokling-Anderson et al., 2009; Villarroya et al., 2014).

Although there were no significance changes in serum adiponectin, previous studies have shown a correlation between n3 PUFA supplementation and an increase in total serum adiponectin (Neschen et al., 2006; Flachs et al., 2006). Neschen et al. (2006) fed 129Sv mice and mice lacking functional PPARα receptors a control diet containing 7% (w/w) fat from soybean oil, as well as diets containing 27% (w/w) safflower oil diet or 27% (w/w) menhaden fish oil. The menhaden oil fed group had plasma adiponectin

levels similar to the control group which was 2.7 fold higher than the safflower oil fed group. They also found an increase in adiponectin gene expression in epididymal fat, but not in the subcutaneous tissue. Furthermore, Flachs et al. (2006) fed C57BL/6J mice diets containing 35% (w/w) fat with low n3 PUFAs or a diet of 35% (w/w) fat containing 0.9% (w/w) EPA and 7.7% (w/w) DHA, and found an increase in plasma adiponectin in the group fed fish oil, similar to mice fed a calorie restricted diet. In contrast Baranowski et al. (2012) found differences in adiponectin levels due to genotype (*fa/fa* Zucker rats versus lean Zucker rats) but no differences due to the amount of dietary ALA present (0-2.8% [w/w]). Riediger et al. (2007) also found no differences in serum adiponectin among C57BL/6 mice fed a diet containing fish oil (0.4% ALA [w/w], 1.3% EPA [w/w] and 0.9% DHA [w/w]), flaxseed oil (3% [w/w] ALA) or safflower oil. Serum resistin was measured in this present study as an indicator of adipocyte dysfunction. In the present study, the faDHA group had reduced body mass, smaller adipocyte size and improvements in insulin resistance but this was not associated with an increase in serum adiponectin as would be expected based on the literature. There were no differences seen in serum resistin seen in any of the groups. Furthermore, serum resistin has not been previously measured in a rodent model alongside an n3 intervention.

SUMMARY AND CONCLUSIONS

Summary of major findings

Generally speaking, *fa/fa* rats fed the diet rich in DHA were closest to the baseline *fa/fa* animals in regards the measured parameters. The marine oil fed groups (EPA and DHA) had the lowest final body weight and total feed consumption among the *fa/fa* experimental animals. Although there were no differences seen in total body fat, the faDHA group showed the most similarities in fat pad mass to the baseline animals after correcting for body weight. The faDHA group maintained a total liver lipid content similar to faBASE. Furthermore, the liver lipid droplets in the faDHA and faEPA groups showed a trend towards smaller lipid droplets compared to the other *fa/fa* animals on experimental diets. This suggests that consumption of DHA in the diet could delay the progression of hepatic steatosis.

The conversion of ALA to DHA was shown with the faALA having the second highest amount of DHA present in the PL fraction after the faDHA group. The faDHA group also had highest total n3 in TAG fraction, and the highest n3 in the PL fraction, which was similar to the faEPA and faALA.

None of the n3 experimental diets had an impact on fasting blood glucose but differences were seen in glucose handling. Although the faLA group peaked at t15, their blood glucose levelled off more efficiently than the faALA group, which remained higher than the faLA group onwards. By t120, faDHA had higher fasting blood glucose than the faLA, lnLA and faBASE, while being similar to faALA and faEPA. This could be due to

the differences in insulin sensitivity present in the groups throughout the OGTT, which was reflected in the serum insulin OGTT values.

The faDHA and faLA groups had the lowest fasting serum insulin among the *fa/fa* experimental groups. They also maintained lower circulating insulin and $AUC_{insulin}$ after glucose load, compared to the other *fa/fa* experimental groups. The HOMA-IR scores of the lnLA were lower than the faALA and faEPA while being similar to the faDHA. Differences between each individual n3 PUFA diet are as follows:

ALA versus EPA

- The faALA group had a higher final body weight and higher total feed intake
- The faALA group had higher total liver lipids and larger lipid droplets
- The faALA group had higher serum AST and ALT levels
- The faALA group had more MUFA and less total n6 in the hepatic TAG fraction
  The faALA group had more ALA, less EPA, less DPA and more DHA in the hepatic PL fraction
- The faALA group exhibited an elevation in hepatic pPPARα
- The faALA group had higher serum total cholesterol, LDL-C and HDL-C
- The faALA had higher serum TAG
- The faALA had more adipocytes in the 2500-3000 μm^2 and >3000 μm^2 ranges than the faEPA

ALA versus DHA

- The faALA group had a higher final body weight and lower total feed intake

- The faALA group had higher total liver lipids and larger lipid droplets
- The faALA group had higher serum AST and ALT levels
- The faALA group had higher levels of serum insulin after a 5 hour fast and during an OGTT and had higher $AUC_{insulin}$
- The faALA group had more total SFA and MUFA than faDHA in the hepatic TAG fraction
- The faALA group had less total n3 in the hepatic TAG fraction. More specifically, the faALA group had more ALA, less DPA and less DHA
- The faALA group had less DGLA in the hepatic TAG fraction
- The faALA group had more ALA and DPA and less DHA in the hepatic PL fraction
- The faALA group had an elevation in pPPARα
- The faALA group had lower circulating haptoglobin
- The faALA group had higher serum total cholesterol, LDL-C and HDL-C
- The faALA group had more adipocytes in the 2500-3000 μm^2 and >3000 μm^2 ranges than the faDHA

EPA versus DHA

- The faEPA and faDHA groups exhibited the lowest body weight among the fa/fa experimental groups, but were not different from each other. The faEPA and faDHA groups also had similar total feed intakes
- The faDHA group had lower hepatic lipid content and had smaller lipid droplets

- The faEPA group had higher levels of serum insulin after a 5 hour fast and during an OGTT
- The faEPA group had less total PUFAs and n3 in the hepatic TAG fraction
- The faEPA group had more EPA and DPA and less DHA in the hepatic PL fraction
- The faEPA group had less LA, GLA and DGLA in the hepatic PL fraction
- The faDHA group had higher circulating haptoglobin
- The faEPA group had lower serum TAG
- Both EPA and DHA groups had similar number of adipocytes in each size range

Overall

Compared to the other experimental diets, the DHA diet attenuated more components related to hepatic steatosis and MetS, which included total liver lipids, lipid droplet size, insulinemia and lipidemia. The DHA diet also prevented the progression of hepatic steatosis as demonstrated by similar total liver lipids to baseline. It should be noted that although DHA attenuated some parameters of hepatic steatosis and MetS, it did not attenuate all of them. Results from this study suggest that all dietary fats and oils play their own individual role in the prevention of the various components of MetS.

The rats fed the ALA diet showed an accumulation of DHA in the hepatic PL fraction higher than that of the EPA diet. Even though there was a higher presence of DHA, the animals fed the ALA did not show similar improvements in MetS parameters or a delay in onset of hepatic steatosis like the DHA fed group. This may suggest that the accumulation of DHA in the tissues may not be the primary reason for the improvements

in MetS parameters, and further research needs to be done to explore the relationship between DHA and its improvement on MetS parameters. This may include research on DHA metabolites such as oxilipins and their pathways.

SIGNIFICANCE

Flaxseed oil is a very rich source of ALA, but due to its high ALA content it is easily susceptible to oxidation and thus unsuitable for frying. It has been proposed that blending flaxseed oil with other oils such as canola would be advantageous in increasing dietary ALA while still retaining properties that would make it suitable for food applications. This study directly compared plant versus marine n3 fatty acid sources on hepatic steatosis, adipose function and MetS characteristics in the same model, which had not been done in the past. This study also explored the potential for ALA to be converted into EPA and DHA and accumulate in the liver.

Since hepatic steatosis is a common consequence of MetS, this present study shows that solely supplementing with DHA could be of potential benefit for patients with MetS and hepatic steatosis. The faDHA group also had the highest amount of total n3 in the hepatic TAG fraction, while exhibiting a low n3/n6 PUFA ratio in the liver. As past studies have showed that patients with hepatic steatosis present a lower n3/n6 polyunsaturated fatty acids ratio in the liver, this further strengthens the potential for DHA supplementation as a benficial supplementation for patients with hepatic steatosis.

EPA was able to attenuate parameters related to MetS and hepatic steatosis, but it was not as effective as the DHA supplementation. Furthermore, the EPA did not provide the same benefit as the DHA in relation to fasting serum insulin, or insulin levels during

the OGTT. The ALA in the diet was not able to benefit any of the measured parameters, and showed the highest degree of hepatic steatosis among the n3 fed groups. Furthermore, ALA created no added benefit in relation to any of the measured MetS parameters.

STRENGTHS

- The first study to directly compare the different chain lengths of n3 PUFAs on hepatic function, adipose function and MetS characteristics.
- The *fa/fa* Zucker rat is a widely used animal model used to study obesity and MetS parameters.
- All the experiemental diets had a macronutrient content representative of the current acceptable macronutrient distribution range for carbohydrate, fat and protein. Furthermore, all of the diets were formulated to have the same amount of calories from each macronutrient source.
- All the diets were formulated to have the same fatty acid composition in relation to SFA, MUFA and PUFA.
- The EPA and DHA were of high purity (>95%) and were analyzed via gas chromatography to confirm their purity.
- The fatty acid profile of the dietary oil mixtures were confirmd by gas chromatography.
- Diet samples were collected throughout the study and analyzed by gas chromatography to ensure that the n3 PUFAs of interest were present in the diet and had not undergone structural changes.

- The rats in this study were still growing and the aim of the study was prevention, therefore, the results of this study may reflect strategies to delay the progression of hepatic steatosis and its consequences in growing children and adolescents.
- There were multiple controls being used which included a baseline control, a low n3 PUFA control in the same obesity model and a lean control. These controls were used to interpret changes due to diet versus changes due to genotype and age.

LIMITATIONS

- The biologically active components of the diets were not identified, therefore, there is no way to ensure that the effects were due to the fatty acids themselves, or due to a metabolic byproduct of the fatty acids and/or oil.
- The n3 PUFA diets compared doses of 5% w/w ALA, 3% w/w EPA and 3% w/w DHA, however, the optimal dose of these n3 PUFAs in this model have yet to be determined.
- The *fa/fa* model used for obesity is due to a genetic mutation in the leptin receptor, which may not accurately reflect the dietary causes of hepatic steatosis in humans.
- All the animals used in this study were male, and there may be sex differences in response to diet.
- While multiple analyses were done to assess insulin resistance, the hyperinsulinemic euglycemic clamp is the gold standard for assessment of insulin resistance.
- Rats are not a good model for cholesterol metabolism in humans.

- The dietary levels of n3 PUFAs given to the experimental animals are unlikely to be obtainable by humans through dietary sources alone.
- The study design did not include a weight matched group fed the LA diet to determine if the lower body weight in the faEPA and faDHA groups contributes to the improvements in the various parameters.
- Flaxseed oil was used as the source of ALA while the EPA and DHA diets used purified forms of EPA and DHA. The processing of the flaxseed oil and/or the presence of minor components could potentially act as confounding variables.
- The ALA diet contained less LA than the EPA and DHA diets.

FUTURE RESEARCH

- To identify and measure the biologically active components in the diet and to further test for changes in oxylipin profile due to the differences in fatty acids consumed in the diets
- Investigate the relationship between improved insulin sensitivity and LA.
- This study only used male *fa/fa* Zucker rats and as sex may play a role in disease progression, a future study comparing the effects of n3 PUFAs on male and female rats should be explored.
- As the diets are rich in a pure n3 PUFA source, additional studies are needed to determine the most effective combination of n3 PUFAs for preventing hepatic steatosis progression and improving adipose function and MetS parameters.

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Appendix 1: Absolute tissue weights

	lnLA	faLA	faALA	faEPA	faDHA	faBASE
Visceral Fat (g)[1]	21.9 ± 1.9^{d}	65.6 ± 2.1^{a}	59.4 ± 2.4^{b}	53.3 ± 2.9^{b}	46.3 ± 2.5^{c}	17.9 ± 0.7^{d}
Epididyimal Fat (g)[1]	6.8 ± 0.5^{c}	18.7 ± 0.8^{a}	15.1 ± 0.8^{b}	14.7 ± 0.8^{b}	13.6 ± 0.8^{b}	5.9 ± 0.3^{c}
Perirenal Fat (g)[2]	10.4 ± 1.6^{e}	30.9 ± 1.1^{a}	27.4 ± 1.8^{ab}	24.6 ± 1.8^{bc}	20.8 ± 1.5^{c}	5.9 ± 0.3^{d}
Mesenteric Fat (g)	6.4 ± 0.5^{d}	16.0 ± 0.6^{a}	16.9 ± 0.7^{a}	14.0 ± 0.8^{b}	11.9 ± 0.7^{c}	6.1 ± 0.3^{d}
Subcutaneous fat (g)[2]	27.6 ± 3.1^{e}	291.9 ± 11.7^{a}	236.8 ± 6.6^{b}	226.0 ± 6.2^{bc}	208.2 ± 7.1^{c}	81.3 ± 5.2^{d}
Total body fat (g)[2]	52.8 ± 3.2^{c}	301.8 ± 11.7^{a}	296.2 ± 8.4^{a}	279.2 ± 7.5^{a}	254.5 ± 9.3^{b}	99.1 ± 5.6^{c}
Liver weight (g)[2]	13.3 ± 0.7^{d}	30.9 ± 2.3^{ab}	32.7 ± 1.2^{2}	28.5 ± 1.2^{bc}	26.0 ± 0.8^{c}	11.0 ± 0.2^{d}

Values are expressed as means ± SEM, n =10 except for faLA, faALA and faEPA where n= 8, 9 and 9, respectively. Different superscript letters indicate significant differences ($p<0.05$) among the means. Total body fat was determined using whole body MRI, visceral fat included epididyimal, perirenal and mesenteric fat pads, and subcutaneous fat was determined by subtracting visceral fat from total fat.

[1] Data was log transformed prior to ANOVA.

[2]Data were analyzed using non-parametric testing for statistical analysis.

Appendix 2: Renal function

A method used to assess decreased renal function is comparing the ratio of serum urea to serum creatinine levels (reviewed by Kim, 2014). Serum was analysed using the cobas c 111 clinical chemistry analyzer (Roche Diagnostics, Indianapolis, IN, USA) for urea (Roche Diagnostics, Indianapolis, IN, cat # 04657527 190) and creatinine (Roche Diagnostics, Indianapolis, IN, cat # 05401755 190). An elevated or reduced serum urea to creatinine ratio is a sign of kidney disorder, and potentially associated with diabetes. This is done by taking a ratio of the two as follows:

$$Urea{:}\,Creatinine\ Ratio = \frac{Serum\ urea\ \left(\frac{mmol}{L}\right)}{Serum\ creatinine\ (\frac{mmol}{L})}$$

Furthermore, serum creatinine and urinary levels of creatinine can be used to measure creatinine clearance which is a method used to determine glomerular filtration rate (GFR), another indication of renal function. The calculation is as follows:

$$Creatinine\ Clearance = \frac{urine\ creatinine\ \left(\frac{mmol}{L}\right) \times urine\ volume\ \left(\frac{mL}{5\ hours}\right)}{serum\ creatinine\ (\frac{mmol}{L})}$$

where 5 hours was expressed in minutes.

Appendix 3: Urinary metabolite excretion

	lnLA	faLA	faALA	faEPA	faDHA	faBASE
Glucose (mmol per 5 hours)	0.86 ± 0.13[b]	21.2 ± 17.3[b]	10.3 ± 8.3[b]	160.6 ± 72.6[a2]	18.3 ± 7.3[b]	0.45 ± 0.1[b]
Urea (mmol per 5 hours)[1]	661 ± 52	683 ± 78	500 ± 54	778 ± 108	697 ± 81	557 ± 83
Creatinine (mmol per 5 hours)	7.13 ± 0.59[a]	5.09 ± 0.47[b]	3.70 ± 0.21[b]	4.30 ± 0.77[b]	4.60 ± 0.70[b]	1.41 ± 0.20[c]
Urea:Creatinine Ratio (mmol per 5 hours: mmol per 5 hours)[1]	92.9 ± 22.7[d]	131.6 ± 11.2 [c]	134.1 ± 12.9[c]	192.1 ± 10.5[b]	158.8 ± 10.7 [c]	394.4 ± 15.0[a]
Creatinine Clearance (mL/min)	22.1 ± 2.6 [E-4]	20.6 ± 1.3 [E-4]	19.3 ± 2.1 [E-4]	18.6 ± 2.8 [E-4]	16.9 ± 2.7 [E-4]	ND

Values are expressed as means ± SEM, n =7-10. Different superscript letters indicate significant differences ($p<0.05$) among the means and an absence of a superscript letter indicates no significant differences.

[1]Data were analyzed using non-parametric testing for statistical analysis.

[2] For the faEPA group, 3 animals had extremely high values (363, 434, 532) and the other 7 animals ranged from 1.22-78.2 (19.1 ± 12.1).

Appendix 4: Urine output

Urine Output (ml/ 5 hours)	lnLA	faLA	faALA	faEPA	faDHA	faBASE
Week 0	0.66 ± 0.2^{c}	5.5 ± 1.7^{ab}	5.16 ± 1.6^{ab}	5.36 ± 0.4^{ab}	5.66 ± 0.5^{a}	4.28 ± 1.1^{b}
Week 4	0.95 ± 0.3^{b}	4.59 ± 1.5^{a}	5.12 ± 1.6^{a}	4.5 ± 0.7^{a}	4.65 ± 0.7^{a}	---
Week 8	1.29 ± 0.4	3.46 ± 0.4	3.87 ± 0.4	3.26 ± 0.6	2.94 ± 0.6	---

Values are expressed as means ± SEM, n =10 except for faLA, faALA and faEPA at week 8 where n= 9. Different superscript letters indicate significant differences ($p<0.05$) among the means and an absences of a superscript letter indicates no significant differences.

Appendix 5: Kidney weights

	lnLA	faLA	faALA	faEPA	faDHA	faBASE
Right kidney (g)	1.59 ± 0.04	2.61 ± 0.52	2.16 ± 0.26	2.10 ± 0.14	2.02 ± 0.11	1.18 ± 0.05
Right kidney (g/100 g BW)	0.34 ± 0.01	0.41 ± 0.09	0.34 ± 0.04	0.36 ± 0.02	0.36 ± 0.02	0.48 ± 0.02
Left kidney (g)	1.52 ± 0.04	2.47 ± 0.49	2.24 ± 0.23	1.97 ± 0.13	2.04 ± 0.01	1.05 ± 0.02
Left kidney (g/100 g BW)	0.36 ± 0.01	0.38 ± 0.09	0.35 ± 0.04	0.33 ± 0.02	0.36 ± 0.02	0.43 ± 0.01

Values are expressed as means ± SEM, n =10 except for faLA, faALA and faEPA where n= 8, 9 and 9 respectively. An absence of a superscript indicates no significant differences.

Appendix 6: Pancreatic immunostaining

Test principle

Insulin immunostaining of the pancreases tissue is used to determine the location of the islets cells and the relative amount of insulin within. Pancreatic sections are rehydrated and then endogenous peroxidase activity is blocked. Samples are incubated within an insulin antibody and then stained with a chromogenic substance. The degree of color development corresponds to the presence of insulin.

Reagents

- STAT-Q Peroxidase-DAB staining System (cat. #314KLD, Innovex Biosciences)
- PAP pen/liquid blocker pen (cat. # MU22, Cedarlane)
- Insulin Mouse Monoclonal Antibody Concentrate (cat. # MAB391C, Innovex Biosciences)
- Background Buster (cat. # NB306, Innovex Biosciences)
- Mayer's hematoxylin
- Permount (cat. # SP15, Fisher Scientific)
- Coverslips, 22×50 premium cover glass (cat. # 12-548-5E, Fisherfinest)

Procedure

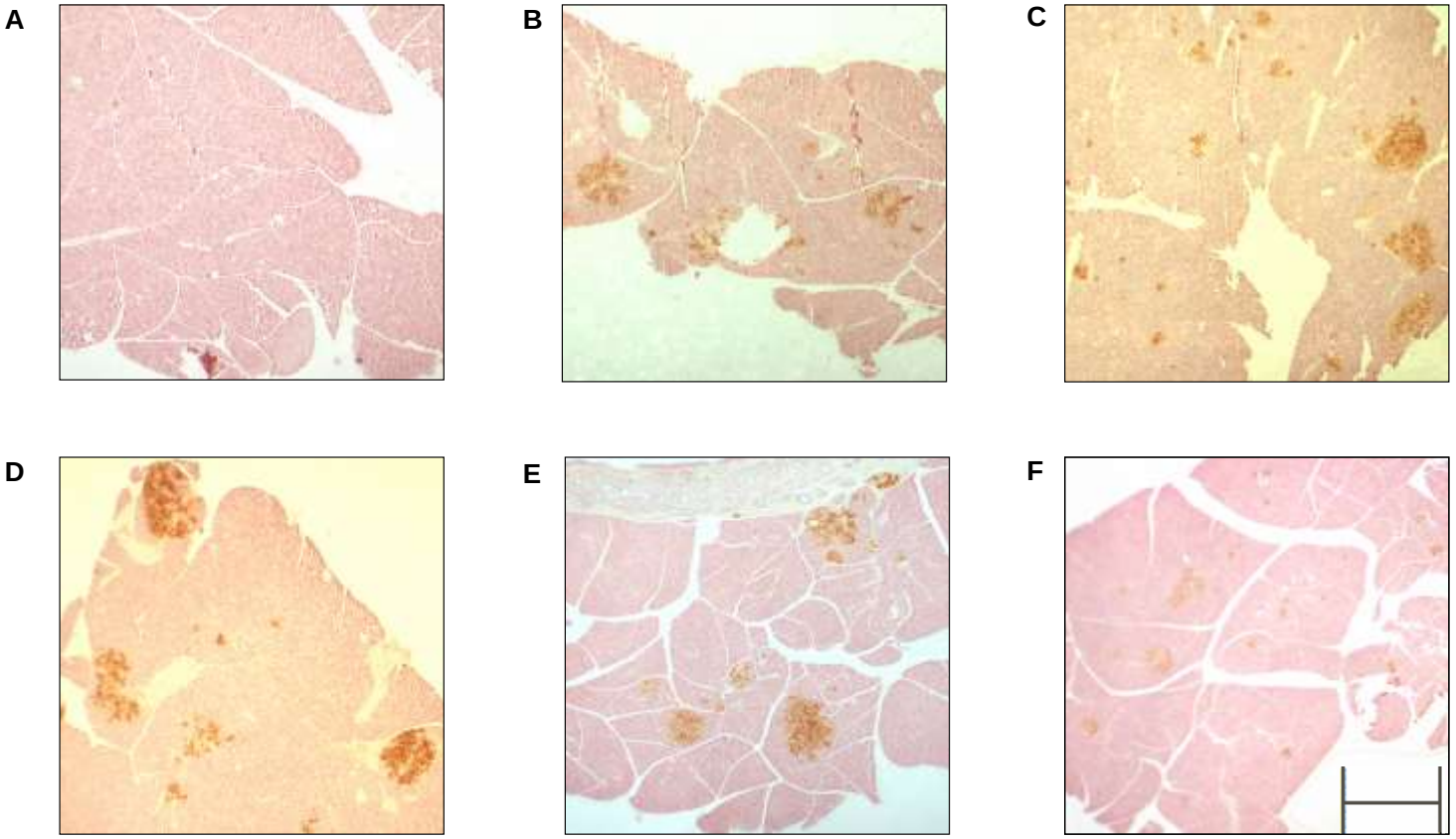
The series of solvents used were in a staining station and the slides were placed in a holder that holds up to 10 slides at a time.

1. The slides were placed in old xylene (cat. # Z33S-4, Fisher Scientific) for 15 minutes. Old xylene has been previously used for this step for up to 45 slides

2. The slides were then placed in new xylene for 15 minutes
3. The tissues were rehydrated by placing the slides in:
    a. 100% ethanol for 3 minutes.
    b. 100% ethanol for 3 minutes
    c. 95% ethanol for 3 minutes
    d. 70% ethanol for 3 minutes
    e. ddH_2O for 5 minutes.
4. After the tissues were rehydrated, endogenous peroxidase activity of the tissue was blocked by placing the slides in fresh 3% H_2O_2 for 10 minutes and washed in 1 × PBS.
5. Slides were removed from PBS and the bottom of the slides along with the area around the tissue section was blotted dry.
6. The PAP pen was used to draw circles around the tissue sections to contain the solutions placed on the tissues.
7. 250 µL of Background Buster was pipette onto each tissue section and the slides were incubated in the incubation chamber (sealable container with a damp paper towel lining the bottom) for 20 minutes.
8. The Background Buster was poured off and the bottom of the slides and the areas surrounding the tissue sections were blotted dry.
9. 250 µL of 1° antibody (insulin antibody) was pipetted onto the tissue section farthest from the slide label. 250 µL of 1 × PBS was pipette on the other tissue (to be used as a control). The slides then incubated for 20 minutes.
10. The slides were then removed from the incubation chamber and rinsed with 1 × PBS.

11. 250 µL of 2° antibody (HRP-labeled Strepavidin) was pipetted onto each tissue. The slides then incubated for 10 minutes in the incubation chamber.
12. The slides were rinsed with 1 × PBS and placed in a fresh a 1 × PBS bath for 5 minutes.
13. While the slides were in the 1 × PBS bath, DAB solution was prepared in the fumehood by mixing 5 mL of DAB substrate with 5 drops of DAB in a vial covered with aluminum foil.
14. The slides were removed from the PBS bath and 250 µL of DAB solution was pipette onto each tissue section in the fume hood. The slides then incubated for 5 minutes.
15. The DAB was rinsed off with ddH_2O into beaker containing 10% bleach.
16. The slides were then counterstained with Mayer's hematoxylin for 1 minute and any excess hematoxylin was rinsed off by dipping slides 3 times in a beaker filled with tap water 3 times.
17. The tissues were dehydrated by placing them in:
    a. 70% ethanol for 3 minutes
    b. 95% ethanol for 3 minutes
    c. 100% ethanol for 3 minutes
    d. 100% ethanol for 3 minutes
18. After the slides were dehydrated they were immersed in new xylene for 3 minutes and allowed to lay flat to dry.
19. Cover slips were mounted onto the sildes by placing a drop of Permount onto the middle of the slide, avoiding the tissue section and gently placing the cover slip over the tissue sections in the middle of the slide. Air bubbles were subsequently removed by pushing down on the cover slip.

20. The slides were dried overnight in the fumehood.
21. Islet cells were visualized under an EVOS^{TM} Core microscope (Fisher Scientific Inc., Ottawa ON). Images were taken at 4× objective magnification and islet size was quantified using ImageJ 1.42 software (National Institute of Health, Bethesda, MD).



Appendix 7: Pancreatic islet insulin immunostaining from lnLA (A), faLA (B), faALA(C), faEPA(D), faDHA(E) and faBASE(F). Representative images of insulin-positive pancreatic islets identified by the brown color from DAB staining. Scale bar equal to 0.05 mm.

Appendix 8: Pancreas function

	lnLA	faLA	faALA	faEPA	faDHA	faBASE
Pancreas weight (g)	1.8 ± 0.1^{a}	1.3 ± 0.1^{b}	1.1 ± 0.1^{b}	1.2 ± 0.1^{b}	1.2 ± 0.1^{b}	1.1 ± 0.1^{b}
Pancreas weight corrected for body weight [1]	0.42 ± 0.02^{a}	0.18 ± 0.01^{b}	0.20 ± 0.01^{b}	0.21 ± 0.01^{b}	0.21 ± 0.02^{b}	0.43 ± 0.03^{a}
Pancreas section: Islet area ratio (mm^2)[2]	0.2 ± 0.04^{c}	3.4 ± 0.9^{a}	3.7 ± 0.6^{a}	1.9 ± 0.6^{ab}	3.4 ± 0.8^{a}	1.0 ± 0.3^{b}

[1]Data was log transformed prior to ANOVA

[2] Data was obtained by taking the area of the islet in the section and dividing it by the area of the pancreas section

Appendix 9: Hepatic cholesterol and TAG procedure

Test principle

Liver samples were lysed and homogenized for analysis of total cholesterol and TAG concentration within the tissues. Total cholesterol (TC, Cholesterol generation 2, Roche Diagnostics, cat. # 04718917 190) and triglycerides (TAG, Roche Diagnostics, Indianapolis, IN, cat. # 04657597 190) were measured using the cobas c 111 clinical chemistry analyzer.

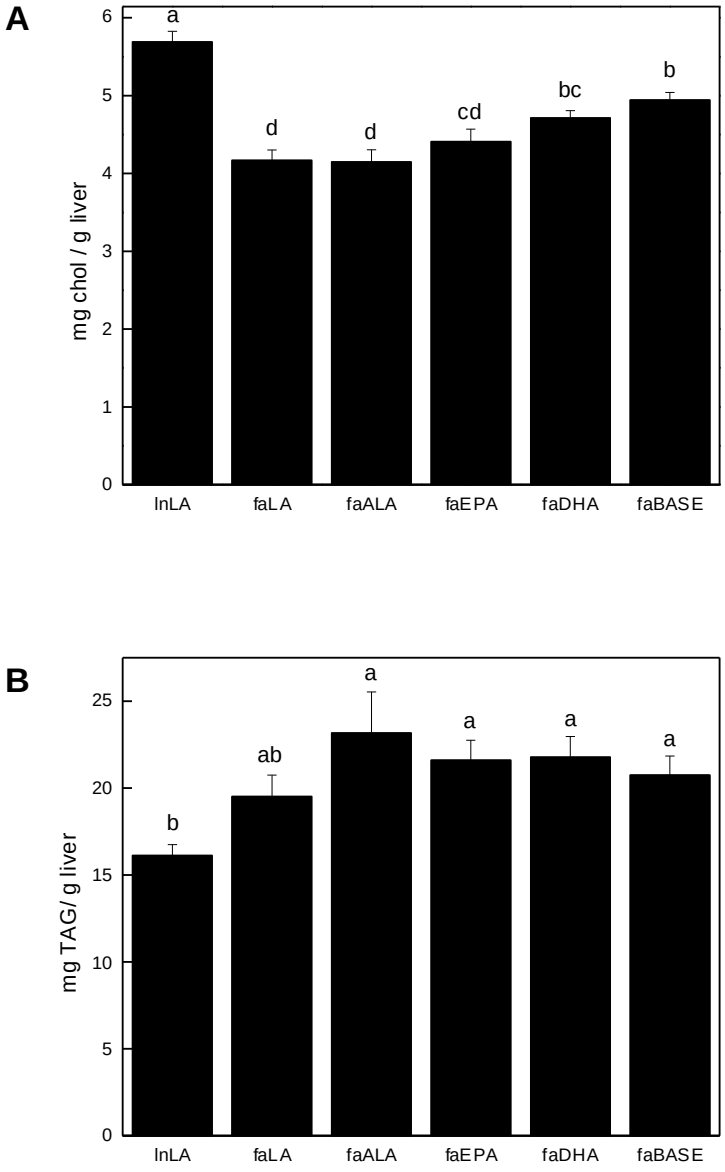
Reagents

- TE buffer with 5% deoxycholate (2 mL of 0.5 mM EDTA [Ethylenediaminetetraacetic acid], 10 mL 1M Tris [pH 7.4], 5 g deoxycholic acid sodium salt, 1 L ddH_2O)

Procedures

1. 0.5 g of thawed liver samples were placed into a 15 mL conical tube containing 10 mL of TE buffer with 5% deoxycholate, and were homogenized for 20 seconds.
2. The samples were then centrifuged at 12000 g for 30 minutes
3. 70 µL of the supernatant was then aliquoted and measured with the cobas c 111 clinical chemistry analyzer.
4. The calculations for mg cholesterol/g of liver and TAG/g of liver are as follows:

$$\frac{mg\ cholesterol}{g\ of\ liver} = \frac{\left(\frac{x}{0.0259}\right)}{10} \times 2 \quad and \quad \frac{mg\ TAG}{g\ of\ liver} = \frac{\left(\frac{x}{0.0113}\right)}{10} \times 2$$



Appendix 10: Hepatic cholesterol (A) and TAG (B). Values are expressed as means ± SEM with n =6-7. Different superscript letters indicate significant differences ($p<0.05$) among the means.

Appendix 11: Hepatic TAG fatty acid composition

Fatty Acid (g/100 fatty acids)	lnLA	faLA	faALA	faEPA	faDHA	faBASE
C16:0[1]	23.1 ± 1.7[d]	38.2 ± 1.4[ab]	37.5 ± 0.8[a]	35.1 ± 0.9[c]	33.1 ± 0.4[c]	40.5 ± 0.5[ab]
C18:0	3.31 ± 0.92	2.39 ± 0.11	3.26 ± 0.24	2.74 ± 0.12	3.32 ± 0.31	4.00 ± 0.25
C18:1n9[2]	15.0 ± 0.7[d]	28.4 ± 1.3[bc]	32.7 ± 0.9[a]	25.7 ± 0.6[c]	26.7 ± 1.8[c]	30.2 ± 1.0[ab]
C18:2[1]	40.6 ± 5.1[a]	16.6 ± 1.5[b]	6.3 ± 0.2[c]	9.5 ± 0.8[c]	11.9 ± 1.2[bc]	10.2 ± 0.6[bc]
C18:3n6[1]	0.914 ± 0.168[a]	0.431 ± 0.027[b]	0.0885 ± 0.1667[c]	0.126 ± 0.008[c]	0.120 ± 0.101[c]	0.283 ± 0.016[bc]
C18:3n3[1]	0.411 ± 0.055[c]	0.188 ± 0.044[c]	5.81 ± 0.32[a]	0.876 ± 0.056[b]	1.00 ± 0.11[b]	0.861 ± 0.083[b]
C20:0	0.0279 ± 0.0031	0.0228 ± 0.0023	0.0266 ± 0.0026	0.0165 ± 0.0012	0.0237 ± 0.0049	0.0255 ± 0.0053
C20:1n9	0.148 ± 0.009	0.0975 ± 0.0200	0.0860 ± 0.0050	0.0678 ± 0.0045	0.0952 ± 0.0174	0.0711 ± 0.0083
C20:2n6[2]	0.343 ± 0.043[a]	0.143 ± 0.028[b]	0.0382 ± 0.0035[e]	0.0508 ± 0.0075[de]	0.0786 ± 0.0127[cd]	0.0875 ± 0.0080[bc]
C20:3n6[1]	0.455 ± 0.067[a]	0.241 ± 0.055[b]	0.101 ± 0.011[c]	0.158 ± 0.019[bc]	0.216 ± 0.028[b]	0.161 ± 0.160[bc]
C20:4n6[2]	6.46 ± 0.79[a]	1.84 ± 0.39[b]	0.27 ± 0.03[c]	0.38 ± 0.05[c]	0.29 ± 0.04[c]	1.10 ± 0.11[b]
C22:1n6[2]	0.0821 ± 0.0261[c]	0.0274 ± 0.0023[c]	0.224 ± 0.022[a]	0.245 ± 0.025[a]	0.111 ± 0.039[b]	0.0197 ± 0.0082[bc]
C20:3n3[1]	0.46 ± 0.30[d]	0.05 ± 0.01[cd]	1.05 ± 0.10[c]	6.44 ± 0.35[a]	1.83 ± 0.27[b]	0.41 ± 0.06[c]

C22:4n6	1.29 ± 0.06[a]	0.87 ± 0.13[b]	0.11 ± 0.01[c]	ND	0.18 ± 0.10[c]	0.71 ± 0.11[c]
C22:5n3[2]	0.47 ± 0.21[c]	0.15 ± 0.04[c]	1.35 ± 0.22[b]	6.28 ± 0.49[a]	2.07 ± 0.23[b]	0.35 ± 0.03[c]
C22:6n3[2]	2.62 ± 2.18[b]	0.21 ± 0.05[b]	1.58 ± 0.21[b]	2.21 ± 0.36[b]	16.4 ± 1.5	0.53 ± 0.07[b]

Values are expressed as means ± SEM, n =6. Different superscript letters indicate significant differences ($p<0.05$) among the means. Only fatty acids with an overall mean >0.05% were reported. ND= not detectable.
[1]Data were analyzed using non-parametric testing for statistical analysis.
[2]Data was log transformed prior to ANOVA.

Appendix 12: Hepatic PL fatty acid composition

Fatty Acid (g/100 fatty acids)	lnLA	faLA	faALA	faEPA	faDHA	faBASE
C16:0[1]	18.4 ± 1.1[a]	19.6 ± 2.4[a]	17.9 ± 0.5[a]	19.8 ± 0.5[a]	20.4 ± 0.4[a]	14.4 ± 0.2[b]
C16:1	0.615 ± 0.360	1.234 ± 0.481	0.809 ± 0.055	0.943 ± 0.066	0.745 ± 0.031	0.786 ± 0.022
C18:0	24.1 ± 1.5	25.8 ± 2.4	26.8 ± 0.7	25.2 ± 0.2	23.5 ± 0.7	28.4 ± 0.3
C18:1n9	3.64 ± 1.55	5.32 ± 2.67	3.38 ± 0.14	3.53 ± 0.10	3.31 ± 0.20	3.05 ± 0.07
C18:1n7c[2]	1.99 ± 0.10[ab]	2.14 ± 0.14[a]	1.64 ± 0.09[c]	1.78 ± 0.06[bc]	1.56 ± 0.03[c]	1.64 ± 0.03[c]
C19:0	0.108 ± 0.008	0.048 ± 0.005	0.028 ± 0.005	0.037 ± 0.003	0.031 ± 0.005	ND
C18:2n6[1]	11.9 ± 0.8[a]	7.7 ± 0.2[c]	9.3 ±0.3[b]	8.3 ± 0.2[bc]	11.5 ± 0.2[a]	9.3 ± 0.2[b]
C18:3n6[2]	0.178 ± 0.021[a]	0.115 ± 0.008[b]	0.079 ± 0.006 [cd]	0.063 ± 0.006 [d]	0.092 ± 0.005[bc]	0.110 ± 0.004[b]
C18:3n3[1]	0.050 ± 0.032[b]	0.091 ± 0.079[b]	0.408 ± 0.026[a]	0.082 ± 0.019[b]	0.058 ± 0.007[b]	0.100 ± 0.009[b]
C20:0	0.0982 ± 0.0059[a]	0.0692 ± 0.0092[b]	0.0517 ± 0.0064[c]	0.0580 ± 0.0026[bc]	0.0455 ± 0.0037[c]	0.0704 ± 0.0041[b]
C20:1n9	0.0937 ± 0.0102	0.0859 ± 0.0107	0.0541 ± 0.0023	0.0533 ± 0.0034	0.0402 ± 0.0041	0.0711 ± 0.0050
C20:2[1]	0.590 ± 0.021[a]	0.292 ± 0.037[b]	0.143 ± 0.006[c]	0.137 ± 0.007[c]	0.174 ± 0.010[c]	0.332 ± 0.010[b]

C20:3n6	0.35 ± 0.02^{c}	0.86 ± 0.07^{c}	2.24 ± 0.10^{a}	1.49 ± 0.05^{b}	2.46 ± 0.14^{a}	1.28 ± 0.13^{b}
C20:4n6[1]	30.0 ± 2.1^{a}	26.5 ± 4.3^{a}	18.4 ± 0.2^{b}	14.7 ± 0.5^{bc}	9.9 ± 0.4^{c}	29.3 ± 0.3^{a}
C20:5n3[1]	0.04 ± 0.01^{c}	0.26 ±0.23^{c}	3.37 ± 0.25^{b}	7.17 ± 0.48^{a}	3.69 ± 0.37^{b}	0.30 ± 0.01^{c}
C22:4n6[1]	1.41 ± 0.26^{a}	0.72 ± 0.17^{a}	0.06 ± 0.07^{b}	0.07 ± 0.01^{b}	0.05 ± 0.01^{b}	0.23 ± 0.02^{b}
C22:5n3[1]	0.41 ± 0.08^{e}	0.51 ± 0.10^{e}	2.13 ± 0.13^{b}	5.37 ± 0.21^{a}	0.94 ± 0.05^{d}	1.38 ± 0.04^{c}
C22:6n3[1]	3.84 ± 0.35^{e}	6.34 ± 0.85^{d}	10.98 ± 0.20^{b}	9.01 ± 0.35^{c}	20.02 ± 0.36^{a}	7.22 ± 0.41^{d}

Values are expressed as means ± SEM, n =6. Different superscript letters indicate significant differences ($p<0.05$) among the means. Only fatty acids with an overall mean >0.05% were reported. ND = not detectable.
[1]Data were analyzed using non-parametric testing for statistical analysis.
[2]Data was log transformed prior to ANOVA.