The Treatment Effects of Dietary Oils on Diet-Induced Obesity, Lipidemia, and Insulin Resistance in Skeletal Muscle Tissue of Obese Prone Rats

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I. ABSTRACT

Reducing consumption of fat is recommended for obese individuals; however, altering dietary fat, without reducing total fat, may modify obesity-associated consequences. The effects of dietary fat composition on obesity and insulin resistance in diet-induced obese rats were investigated. Rats were fed a high-fat lard-based diet for 12 weeks and then were randomized into one of six high-fat treatment groups (oils used: high-oleic canola, conventional canola, high-oleic/conventional canola mix, conventional canola/flax mix, safflower, or soybean) or kept on the lard diet for 8 weeks. Diets had varying effects on lipidemia and glycemia; however, insulin tolerance tests, oral glucose tolerance tests, and the skeletal muscle response to insulin were not different among groups. Muscle phospholipids showed expected differences in fatty acid (FA) composition, but polyunsaturated/saturated FA ratios were not different among groups. Overall, a consistent response to high-fat diets was observed which may be attributed to the robustness of polyunsaturated/saturated FA ratios of muscle phospholipids.

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VII. LIST OF ABBREVIATIONS AND TERMINOLOGY

AA	Arachidonic acid
AIN	American Institute of Nutrition
AIN-93G	AIN-93 standardized rodent diet for growth
AIN-93G-MX	AIN-93 standardized rodent mineral mix for growth
AIN-93-VX	AIN-93 standardized rodent vitamin mix
ANOVA	Analysis of variance
ALA	Alpha-linolenic acid
AMDR	Acceptable macronutrient distribution range
AUC_G	Area under the curve for glucose
AUC_{GI}	Glucose insulin index
AUC_I	Area under the curve for insulin
β-ME	2-mercaptoethanol
BCA	Bicinchoninic acid
BHT	2,6-Di-*tert*-butyl-4-methylphenol
blength	Body length
BMI	Body mass index
BPB	Bromophenol blue
BSA	Bovine serum albumin
bwt	Body weight
C	Conventional canola oil group
CDA	Canadian Diabetes Association
CF	Conventional canola/flax oil group

CM	Conventional canola/high-oleic canola oil (canola mix) group
Canola-based groups	HC, C, CM, CF
ddH_2O	Double distilled water
DHA	Docosahexaenoic acid
DIO	Diet-induced obese
DM-2	Type 2 diabetes mellitus
EPA	Eicosapentaenoic acid
FA	Fatty acid
FFA	Free fatty acid
GC	Gas chromatography
HC	High-oleic canola oil group
HOMA-IR	Homeostatic assessment model for insulin resistance
HRP	Horseradish peroxidase
ITT	Insulin tolerance test
IRS	Insulin receptor substrate
L	Lard group
L-12	Lard group terminated at week 12
L-20	Lard group terminated at week 20
LA	Linoleic acid
LF	Low fat group
LF-12	Low fat group terminated at week 12
LF-20	Low fat group terminated at week 20
MUFA	Monounsaturated fatty acid

Muscle	Skeletal muscle
n-3	3 carbon atoms from the methyl end of a FA hydrocarbon chain
n-6	6 carbon atoms from the methyl end of a FA hydrocarbon chain
n-9	9 carbon atoms from the methyl end of a FA hydrocarbon chain
OA	Oleic acid
OGTT	Oral glucose tolerance test
OP	Obese prone
OR	Obese resistant
PIP_2	phosphatidylinositol-4,5-bisphosphate
PIP_3	phosphatidylinositol-4,5-trisphosphate
PI3-kinase	Phosphatidylinositol 3-kinase
PL	Phospholipid
PUFA	Polyunsaturated fatty acid
PVDF	Polyvinylidene diflouride
SAPK/JNK	Stress activated protein kinase/c-Jun N-terminal kinase
SB	Soybean oil group
SDS-PAGE	Sodium dodecylsulfate polyacrylamide gel electrophoresis
Ser^{473}	Serine phosphorylation at site 473
SF	Safflower oil group
SFA	Saturated fatty acid
Short-chain n-3	Short-chain n-3 PUFA (contains 18 carbon atoms)

TBST	Tris-buffered saline in tween-20
TEMED	N,N,N',N'-Tetramethylethylenediamine
Thr^{183}	Threonine phosphorylation at site 183
Thr^{308}	Threonine phosphorylation at site 308
TG	Triglyceride
TQ	Trace quantity
TLC	Thin-layer chromatography
Tyr^{185}	Tyrosine phosphorylation at site 185
VLC	Very long chain (contains 20-22 carbon atoms)
WAT	White adipose tissue
WC	Waist circumference

NB: Abbreviations followed by a lower-case "s" in thesis indicates that the abbreviated word is plural

VIII. LITERATURE REVIEW

Introduction

Obesity and type 2 diabetes (DM-2) are health concerns of epidemic proportions. In 2007-2009, approximately 24% of Canadian adults were obese and the prevalence of obesity has significantly increased over the last 20 years (Shields *et al*., 2011). Overweight and obesity are major risk factors for developing DM-2 and DM-2 contributes to approximately 90% of all diagnosed cases of diabetes (Mahan & Escott-Stump, 2004). In Canada, approximately 9 million people have diabetes or pre-diabetes and about 41,500 Canadians die from diabetes-associated complications each year; it is estimated that by the year 2020, diabetes will cost the Canadian healthcare system $16.9 billion per year (Canadian Diabetes Association, 2011). It is apparent that reducing the prevalence of obesity, or at least the consequences associated with it, is highly desirable.

The causes of obesity are multi-factorial and not well defined, however, genetics and lifestyle are two areas often investigated in regards to factors affecting obesity development. It has been proposed that typical obesity is, in part, inherited; there is not one major gene that causes obesity, rather, there are several common gene variants that play a role, indicating that obesity is polygenic in origin (Mahan & Escott-Stump, 2004). Furthermore, high energy or high fat diets have been implicated as contributing factors to obesity (Mahan & Escott-Stump, 2004), while reducing calories and fat consumption is recommended as a lifestyle intervention for obese individuals (DeClercq *et al*., 2008). Consequences of obesity, due to altered body composition, result in a number of metabolic changes which are reflected in parameters at whole body, specific tissue, and molecular levels. Overall, these changes contribute to insulin resistance which is a

preliminary step in the development of DM-2 (Mahan & Escott-Stump, 2004).

Current recommended prevention strategies for DM-2 include moderate weight loss through healthy eating and regular physical activity, and, in some cases, use of pharmacological agents (Canadian Diabetes Association, 2008). Management of the disease involves self-monitoring of blood glucose levels, ketone testing if symptoms of diabetic ketoacidosis are present, 150 minutes of moderate intensity aerobic activity per week, resistance activity 3 times per week, counselling by a registered dietitian regarding following Canada's Food Guide and choosing low glycemic index foods, and use of pharmacological agents and insulin (Canadian Diabetes Association, 2008).

Although healthy eating, physical activity, and weight loss are of primary importance for prevention and management of both obesity and DM-2, there is evidence to suggest that consequences of these conditions can be minimized by changing the composition of fat consumed rather than reducing the amount of total fat in the diet.

Obesity and Insulin Resistance at the Whole Body Level

Determinants of Obesity

Obesity is a condition of excessive fatness leading to health risks and can be measured, in humans, in two main ways: body mass index (BMI), and waist circumference (WC) (Douketis *et al.*, 2005). BMI is a measure of weight (in kilograms) divided by height (in meters) squared and a BMI greater than 30 kg/m^2 is classified as obese (Douketis *et al*., 2005). Furthermore, BMI levels are positively associated with risks of developing health problems such as DM-2 (Douketis *et al*., 2005). BMI is a relatively easy measurement to obtain, but it is not accurate as it does not take body

composition into account. For example, BMI can misclassify people with large muscle mass as obese even though their body fat percentage is low. Therefore, WC should also be measured when determining obesity. A WC ≥ 102 cm in men, and ≥ 88 cm in women, is associated with increased risk of health problems including DM-2 (Douketis *et al*., 2005).

Obesity and Insulin Resistance

As mentioned, obesity is a major risk factor for developing DM-2. The exact reasons for development of insulin resistance in obesity are numerous and not well defined, however, a simplified explanation of the progression from obesity to DM-2 is given here. First, obesity results in enlarged fat mass and usually dyslipidemia (Sizer & Whitney, 2006). Dyslipidemia, especially elevated free fatty acids (FFAs) in the blood, causes impairment of the insulin signalling cascade (Belfort *et al*., 2005) and preliminary insulin resistance occurs. Insulin resistance worsens as more FFAs are released into the bloodstream and blood glucose and insulin levels increase (Mahan & Escott-Stump, 2004). "Prediabetes" occurs when blood glucose levels are significantly elevated but not to the level of diagnosis for diabetes (Canadian Diabetes Association, 2008). Finally, blood glucose levels become elevated enough to reach the diagnostic criteria for diabetes (Canadian Diabetes Association, 2008).

The ways in which insulin resistance causes dysfunction in the processes that are controlled by insulin at the whole body level, namely carbohydrate and fat metabolism, are outlined below. Changes to these processes are reflected in blood levels of glucose, insulin, triglycerides (TGs), and FFAs.

a) Carbohydrate metabolism

The transport of glucose into cells is probably the most recognizable function of insulin, but insulin also prevents breakdown of glycogen and promotes glucose storage in muscle and the liver (Mahan & Escott-Stump, 2004). In this way, regular insulin function is of immense importance for proper carbohydrate metabolism. At the whole body level, effects of insulin resistance on carbohydrate metabolism are most easily measured by blood glucose concentrations (since blood glucose levels rise in insulin resistance) during an oral glucose tolerance test (OGTT). The diagnostic criteria for diabetes are fasting plasma glucose of $\geq$ 7.0 mmol/L or plasma glucose (measured two hours after administration of 75 g of glucose) $\geq$ 11.1 mmol/L (Canadian Diabetes Association, 2008). Complications of the chronic hyperglycemia associated with DM include damage, dysfunction, and failure of kidneys, eyes, nerves, heart, and blood vessels (Canadian Diabetes Association, 2008). Hyperinsulinemia is also a measurable indication of insulin resistance (Mahan & Escott-Stump, 2004).

b) Lipid Metabolism

Similar to carbohydrate metabolism, insulin affects lipid metabolism by increasing uptake of TGs, preventing lipolysis, and promoting lipogenesis (Mahan & Escott-Stump, 2004). When insulin resistance occurs, plasma TGs increase due to reduced activation of lipoprotein lipase (Canadian Diabetes Association, 2008). Furthermore, FFAs and glycerol levels in the blood increase due to reduced inhibition of hormone sensitive lipase (Mahan & Escott-Stump, 2004). Elevated lipid levels contribute to the complications of DM. In particular, the prevalence of coronary artery disease is 2-3 times higher in people with DM than those without DM, and coronary/cerebrovascular

events account for >75% of deaths in people with DM (Canadian Diabetes Association, 2008). In terms of blood lipid targets for people with diabetes, there are no current recommendations for FFAs or TGs, but, a plasma TG level <1.5mmol/L is considered optimal (Canadian Diabetes Association, 2008).

Obesity and Insulin Resistance at the Tissue Level

Skeletal Muscle Response

Skeletal muscle tissue is the main site for insulin-stimulated glucose use (Kelley *et al.*, 2002); therefore, examining insulin resistance at this level is of primary importance. The FA composition of skeletal muscle phospholipids (PLs) plays an important role in insulin resistance.

It has been shown that an increase in long chain polyunsaturated fatty acids (PUFAs) in muscle PLs is related to a reduction in serum insulin concentration indicating a relationship between long chain PUFAs and insulin sensitivity (Borkman *et al*., 1993). Conversely, levels of linoleic acid (LA) are positively correlated with serum insulin levels (Borkman *et al*., 1993). Although the mechanism by which PL composition affects insulin resistance is unclear, is has been hypothesized that FAs may have an effect on membrane proteins such as insulin receptors as these are in direct contact with the PL bilayer (Borkman *et al*., 1993).

Obesity and Insulin Resistance at the Molecular Level in Muscle Tissue

Insulin Signalling Cascade

The insulin signalling cascade is a complex series of events, largely centered on

the phosphorylation of intracellular proteins, that begins with insulin binding to the extracellular domain of the transmembrane insulin receptor and ends with a number of different outcomes including gene regulation, growth, differentiation, glycogen and protein synthesis, and glucose uptake into the cell (Glund & Zierath, 2005); dysfunction of insulin signalling is the basis for insulin resistance. Akt and stress activated protein kinase/c-Jun N-terminal kinase (SAPK/JNK) are two key proteins involved in the insulin signalling cascade.

a) Akt

The first few steps in the insulin signalling cascade include tyrosine phosphorylation of the insulin receptor then insulin receptor substrate (IRS). After IRS is phosphorylated, it binds to and activates phosphatidylinositol 3-kinase (PI3-kinase). PI3-kinase phosphorylates phosphatidylinositol-4,5-bisphosphate (PIP_2) to form phosphatidylinositol-3,4,5-trisphosphate (PIP_3). PIP_3 then recruits serine-threonine kinases including Akt. Akt has many phosphorylation sites; in order for Akt to become activated, serine-473 (Ser^{473}) and threonine-308 (Thr^{308}) must be phosphorylated (Persad *et al*., 2001). Akt activity is reduced in muscle tissue of people with DM-2 (Glund & Zierath, 2005).

b) SAPK/JNK

SAPK/JNK is a protein that inhibits insulin signalling by promoting phosphorylation of IRS at serine-307 (Lee *et al.*, 2003). Chronic inflammation (such as what occurs in obesity) is one of the ways in which SAPK/JNK can become activated (Lee *et al*., 2003). In muscle tissue of obese rodents, SAPK/JNK activity is elevated; when whole body SAPK/JNK is deficient in mouse models of obesity, adiposity is

reduced and insulin sensitivity is increased (Chen, 2006; Hirosumi *et al*., 2002). However, when SAPK/JNK is selectively deleted in skeletal muscle tissue of high-fat fed mice, obesity is not-altered but insulin sensitivity is improved (Sabio et al., 2010). For SAPK/JNK to become activated, it must be phosphorylated at threonine-183 (Thr^{183}) and tyrosine-185 (Tyr^{185}).

Dietary Fats

Fat is a necessary component of the human diet as it is a major source of energy and aids in the absorption of fat soluble vitamins. Additionally, it is recommended that adult humans consume approximately 20-35% of energy as fat (Trumbo *et al.*, 2002). Fats are primarily consumed as TGs; TGs consist of a glycerol molecule esterified with three FA molecules. All FAs are hydrocarbon chains, of varying length and saturation, with a methyl end and a carboxyl end. The major FAs in a typical human diet are saturated FAs (SFAs), monounsaturated FAs (MUFAs) and PUFAs. SFAs contain no double bonds between carbon atoms, MUFAs contain one double bond, and PUFAs contain two or more double bonds. Unsaturated FAs (MUFAs and PUFAs) are classified by where the double bond closest to the methyl end of the hydrocarbon chain occurs; n-9 MUFAs, n-6 PUFAs, and n-3 PUFAs have double bonds on the 9^{th}, 6^{th}, and 3^{rd} carbon atom from the methyl end, respectively (Institute of Medicine of the National Academies, 2002). Table 1 shows structures of common FAs.

The body can synthesize SFAs and n-9 MUFAs, however, LA (an n-6 PUFA) and α-linolenic acid (ALA, an n-3 PUFA) are essential in the diet and deficiency symptoms develop if they are not consumed. LA is converted in the body to arachidonic acid (AA)

Table 1. Common fatty acids

Notation[1]	Common Name	Formula[2]
Saturated Fatty Acids		
14:0	Myristic acid	CH_3-$(CH_2)_{12}$-COOH
16:0	Palmitic acid	CH_3-$(CH_2)_{14}$-COOH
18:0	Stearic acid	CH_3-$(CH_2)_{16}$-COOH
Monounsaturated Fatty Acids		
18:1 (n-9)	Oleic acid	CH_3-$(CH_2)_7$-CH=CH-$(CH_2)_7$-COOH
Polyunsaturated Fatty Acids		
18:2 (n-6)	Linoleic acid	CH_3-$(CH_2)_4$-CH=CH- CH_2-CH=CH-$(CH_2)_7$-COOH
18:3 (n-3)	α-Linolenic acid	CH_3-$(CH_2$-CH=CH$)_3$-$(CH_2)_7$-COOH
20:4 (n-6)	Arachidonic acid	CH_3-$(CH_2)_3$-$(CH_2$-CH=CH$)_4$-$(CH2)_3$-COOH
20:5 (n-3)	Eicosapentaenoic acid	CH_3-$(CH_2$-CH=CH$)_5$-$(CH_2)_3$-COOH
22:6 (n-3)	Docosahexaenoic acid	CH_3-$(CH_2$-CH=CH$)_6$-$(CH_2)_2$-COOH

[1]The first number indicates the amount of carbons in the hydrocarbon chain; the second number indicates the number of double bonds; numbers in parentheses indicate where the double bond occurs in relation to the methyl end of the hydrocarbon chain.
[2]CH_3 indicates the methyl end of the hydrocarbon chain; COOH indicates the carboxyl end of the hydrocarbon chain; - indicates single bonds; = indicates a double bonds.
Adapted from Gropper *et al.*, 2005.

which plays a role in gene expression. Similarly, ALA is converted to eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA); EPA is recognized as being beneficial for protecting against coronary heart disease, arrhythmias, and thrombosis (Institute of Medicine of the National Academies, 2002). Furthermore, both AA and EPA are precursors for eicosanoids; eicosanoids have many actions including affecting blood pressure and blood platelet aggregation (Gropper *et al.*, 2005).

In order for LA to be converted AA and ALA to be converted to EPA and DHA, they must go through a series of elongation and desaturation processes (Figure 1). LA and ALA share the same elongase and desaturase enzymes so a proper ratio of LA: ALA must be consumed in order to balance the competition for these enzymes and ensure adequate amounts of AA, EPA, and DHA are synthesized; for this reason it has been estimated that a ratio of 5:1-10:1 (LA:ALA) is optimal (Institute of Medicine of the National Academies, 2002).

It has been shown that in diet-induced models of obesity employing a high fat diet, the FA profile of the diet may have effects on parameters associated with obesity and insulin resistance. Additionally, the Canadian Diabetes Association (CDA) recommends consuming meals that favour MUFAs and n-3 PUFAs over other types of fats (Canadian Diabetes Association, 2008). A comparison of dietary fats is shown in Table 2. Briefly, fats that are high in n-3 PUFAs are flax (high in ALA) and fish oils (high in EPA and DHA); lard is high in SFAs and contains moderate amounts of MUFAs; olive oil is high in MUFAs; safflower oil is high in n-6 PUFAs (Canola Council of Canada, 2009a). In order to meet the CDA recommendations, consumption of olive oil and flax/fish oils seems like the obvious choice. However, olive oil contains very low

amounts of n-3 PUFAs and flax/fish oils are highly unsaturated making them susceptible to oxidation. Oils that are susceptibility to oxidation have shortened shelf-life, poor stability, and are unsuitable for cooking.

Canola oil is suitable for baking, stir frying, and deep frying as there is no significant development of *trans* fats or loss of unsaturated FAs during heating (Canola Council of Canada, 2009a). Canola oil is composed of approximately 7% SFAs, 21% LA, 11% ALA, and 61% oleic acid (Canola Council of Canada, 2009a). This means that it is relatively low in SFAs, high in MUFAs, intermediate in PUFAs with a good ratio of n-6 to n-3 PUFAs. There is also "high-oleic" canola oil on the market which is more stable than conventional canola oil and is predominantly used in commercial food production; its composition consists of significantly more MUFAs (70%) than conventional canola oil which makes it more similar to olive oil (Canola Council of Canada, 2009b) (Table 2 shows fatty acid content of select dietary fats). Interestingly, there have been few investigations into the effects of canola oil on parameters associated with obesity and insulin resistance. Furthermore, due to its unique and complex composition, it is impossible to predict the effects it may have.

The following sections were formed by a literature review of the current body of knowledge related to effects of dietary FA composition on parameters of obesity and insulin resistance. Where possible, the information provided was limited by selecting studies that used plant sources of n-3 PUFAS in their dietary interventions; in areas where research using plant-based n-3 PUFAs was lacking, studies that tested other fat sources (namely fish oils) were included. The review of literature was also limited by the models used; studies using rodent models were preferentially reviewed, however, human

studies and cell models were also included when rodent studies provided limited or no information in a given area.

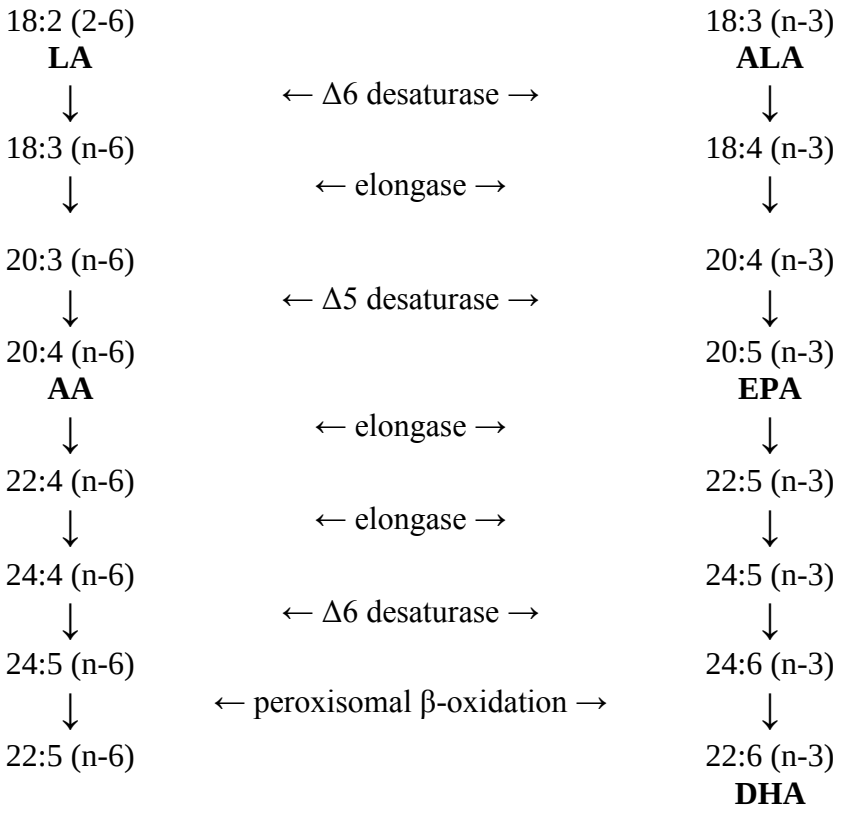


Figure 1. Desaturation and elongation of linoleic acid (LA) to arachidonic acid (AA) and α-linolenic acid (ALA) to eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). Desaturase enzymes create double bonds between carbon atoms; the doubles bonds are placed at the specified carbon closest to the carboxyl end of the hydrocarbon chain (for example, a Δ 6 desaturase enzyme will create a double bond between carbons 6 and 7 from the carboxyl end of the hydrocarbon chain). Elongase enzymes increase the hydrocarbon chain length by adding 2 carbon atoms at the carboxyl end. Adapted from Institute of Medicine of the National Academies, 2002 and Sprecher, 2000.

Table 2. Fatty acid composition of select dietary fats[1]

Dietary Fat	% SFA	% n-6 PUFA (LA)	% n-3 PUFA (ALA)	% MUFA (OA)
Canola Oil	7	21	11	61
High-Oleic Canola Oil	7	20	3	70
Flaxseed Oil	9	16	57	18
Lard	43	9	1	47
Olive Oil	15	9	1	75
Safflower Oil[2]	9	75	0.3	16
Soybean Oil	15	54	8	23
Sunflower Oil	12	71	1	16
Corn Oil	13	57	1	29

[1]LA, Linoleic acid; ALA, α-linolenic acid; OA, oleic acid.
Adapted from Canola Council of Canada (2009a)
[2]In the Canola Council of Canada (2009a), document high-oleic safflower fatty acid composition is listed. Here, a high-linoleic safflower oil composition is given based on an average of values from Fasina *et al.* (2008) and Rice *et al.* (2000).

Dietary Fatty Acid Composition and Parameters at the Whole Body Level

Determinants of Obesity

Obesity can be determined by BMI and WC in humans. In animal models, such measurements do not apply, but measuring weight gain and white adipose tissue (WAT) accumulation can provide insights into the development of obesity in these models. A 19-week study by Ikemoto *et al*. (1996) compared how several different fats affected overall weight gain and WAT weight in 7-week old female C57BL/6J mice. The diets were high in fat (60% of total calories) and were classified by the fat used: soybean oil (high n-6 plus ALA), lard, rapeseed oil, safflower oil, perilla oil (high in ALA), and fish oil; it is assumed here that the rapeseed oil used was a low erucic acid variety, also known as canola oil. All fats, except fish oil, contributed to significant overall weight gain, over 18 weeks, compared to the control (high carbohydrate) diet. The soybean diet contributed to the most overall weight gain; lard, rapeseed, and safflower diets resulted in moderate weight gain; perilla diet showed modest, but significant, weight gain. WAT weight followed a similar pattern to overall weight gain except soybean oil was comparable to lard, rapeseed oil, and safflower oil.

A study by Okuna *et al*. (1997), also evaluated the effects of fat manipulation on obesity. For their study, 4-week old Sprague-Dawley rats were fed for 12 weeks on 1 of 4 diets; lard (beef tallow), olive oil, safflower oil, or perilla oil were the sources of fat in these diets and all diets contained 26.2% of energy from fat. At the end of the study, none of the groups differed in body weight or feed intake, however, the perilla group had significantly lower epididymal fat pad mass compared to the lard and olive oil groups (the safflower group was in between and did not differ from any group). No differences

were seen in peri-renal fat pad mass among groups.

Insulin Resistance

a) Carbohydrate Metabolism

Storlein *et al*. (1991) have used a variety of dietary fats to determine the effects of FA composition on parameters associated with obesity and insulin resistance in 8-week old male Wistar rats. The diets included high SFA (mix of safflower oil and lard), high MUFA (olive oil), high n-6 PUFA (safflower oil), n-6 PUFA + very long chain (VLC) n-3 PUFA (mix of safflower and fish oils), n-6 PUFA + short chain n-3 PUFA (mix of safflower and flaxseed oil), SFA + short chain n-3 PUFA (mix of lard and flaxseed oil), and a high-carbohydrate control diet. Note that, in this study, VLC PUFAs are defined as containing 20-22 carbon atoms while short-chain PUFAs contain 18 carbon atoms. The diets without n-3 PUFAs contained fat as 59% of total calories while diets with n-3 PUFAs contained fat as 65% total calories. The study lasted for 4 weeks. Whole body insulin action (determined by hyperinsulinemic euglycemic clamp) was severely impaired by the SFA, MUFA, n-6 PUFA, and n-6 PUFA + short chain n-3 PUFA diet. Rats fed the n-6 PUFA + VLC n-3 PUFA and SFA + short chain n-3 PUFA diets were more insulin sensitive compared to those on the control diet. It was suggested that the competition of LA and ALA for the same desaturation and elongation enzymes resulted in a lesser effect of ALA in the n-6 PUFA + short chain n-3 diet compared to the SFA + short chain n-3 PUFA diet; the ratio of LA:ALA in the n-6 PUFA + short chain n-3 diet was approximately 10.5:1 which is slightly higher than the recommended range, for humans, of 5:1 to 10:1 (Institute of Medicine of the National Academies, 2002). The ratio of LA:ALA in the SFA + short chain n-3 diet was approximately 0.8:1.

Insulin sensitivity has also been assessed by OGTT. In the aforementioned study by Ikemoto *et al*. (1996), all groups showed significant increases in blood glucose concentrations at 30, 60, and 120 minutes of an OGTT, after 3 weeks of diet intervention compared to the high-carbohydrate control. After 18 weeks, blood glucose (30 minutes after a glucose load) was highest in the safflower oil group, moderately elevated in the lard, soybean oil, and rapeseed oil groups, and mildly elevated in the perilla and fish oil groups compared to the high-carbohydrate control.

A study by Mori *et al.* (1997) also assessed insulin sensitivity by OGTT but used 8-week old male Otsuka Long-Evans Tokushima Fatty rats, a model of spontaneous DM-2 with obesity. Rats were supplied with "MB-3 rat chow" and tap water *ad libitum* for the duration of the study (18 weeks). Daily doses of 0.3 g/kg body weight of the test fats (lard, olive oil, safflower oil, and highly purified EPA ethyl ester) were provided via gavage; the control substance was 0.3 mL/kg/d of distilled water. Rats on the lard, olive oil, and safflower oil interventions showed significantly higher blood glucose concentrations (at 0 and 120 minutes of the OGTT) compared to the control group. Blood glucose levels of the EPA ethyl ester group did not differ significantly from the control group at 0, 60 and 120 minutes of the OGTT.

Another method of measuring insulin sensitivity is the insulin tolerance test (ITT). Mustad *et al*. (2006) used this method in their 4-week study with 4-week old, male, *ob/ob* mice. Mice were fed one of several high fat (43-45% of energy) diets; the two diets of interest here were the control diet (fat composition: 85% high-oleic safflower oil, 10% canola oil, 5% lecithin) and the ALA blend diet (fat composition: 50% high-oleic safflower oil, 39% flaxseed oil, 6% corn oil, and 5% lecithin). A 2-hour ITT showed that

insulin sensitivity was significantly higher in the ALA blend group compared to the control group.

b) Lipid Metabolism

Lipid metabolism is altered in insulin resistance resulting in altered blood lipid profiles. The study by Mori *et al*. (1997) (previously mentioned) found significant increases in fasting (4 hour) plasma TGs in the lard and olive oil intervention groups compared to the safflower and purified EPA ethyl ester diets (which did not differ significantly from the control diet). Additionally, all intervention groups had significantly increased fasting (4 hour) plasma FFAs compared to the control.

The study by Mustad *et al*. (2006) (previously mentioned) found that the ALA blend diet resulted in significantly lower plasma TGs compared to the control diet.

A study by Jeffery *et al.* (1996) compared the effects of diets with various n-6:n-3 PUFA ratios on serum TGs. Three week old male Lewis rats were used and the study lasted for 6 weeks. Diets contained 40% of energy from fat and the sources of fat were sunflower and linseed oil. By using various amounts of sunflower and linseed oil, 5 different diets were developed: diet A, 100% sunflower oil (n-6:n-3 = 112.5:1); diet B, 94% sunflower oil and 6% linseed oil (n-6:n-3 = 14.75:1); diet C, 88% sunflower oil and 12% linseed oil (n-6:n-3 = 6.47:1); diet D, 37% sunflower oil and 63% linseed oil (n-6:n-3 = 0.82:1); diet E, 100% linseed oil (n-6:n-3 = 0.33:1). Non-fasted serum TGs were similar in groups A, B, and C but groups D and E has significantly lower TGs compared to groups A, B, and C. Overall, there was a pattern of reductions in serum TGs with reductions in the n-6:n-3 PUFA ratio of the diet.

Dietary Fatty Acid Composition and Parameters at the Tissue Level

Skeletal Muscle Response

The composition of PLs in muscle tissue is related to insulin action; Storlein *et al*. (1991) found that EPA and DHA in muscle PLs were positively associated with insulin sensitivity. They also found that n-3 PUFAs in the diet were related to EPA and DHA levels in muscle PLs. Similar to other findings in their study, the diets containing n-6 PUFA + VLC n-3 PUFA and SFA + short chain n-3 PUFA were associated with the highest levels of EPA and DHA in muscle PLs. This also adds support to the idea that diets high in n-6 PUFAs inhibit conversion of ALA to EPA and DHA since the n-6 PUFA + short chain n-3 PUFA diet resulted in significantly less EPA and DHA in muscle PLs than the other n-3 PUFA containing diets. Mori *et al*. (1997) also showed that diet can alter muscle PL composition. Their purified EPA ethyl ester diet was associated with significantly more LA and EPA in muscle PLs compared to the lard, olive oil, and safflower oil diets; DHA levels were not significantly different among diets. LA levels were higher in the purified EPA ethyl ester group probably because EPA has an inhibitory effect on desaturation and elongation of LA to AA (Mori *et al*., 1997). Although LA in muscle PLs is associated with insulin resistance (Borkman *et al*., 1993), this study showed that insulin sensitivity was maintained with the purified EPA ethyl ester diet.

Dietary Fatty Acid Composition and Parameters at the Molecular Level

a) Akt

Insulin-stimulated Akt activation in the presence of different FAs was

investigated in a study by Chavez and Summers (2003). C2C12 myotubes (at 4 days after differentiation) were treated with 0.75 mM FFAs for 16 hours. The FFAs were palmitate (a SFA) and oleate (a MUFA). By use of immunoblot analysis it was determined that in the presence of insulin, cells treated with palmitate showed lower Akt-Ser^{473} phosphorylation than those treated with oleate. The cells treated with oleate had similar Akt-Ser^{473} phosphorylation to the untreated control.

Le Foll *et al*. (2007) investigated the effects of n-3 PUFAs on Akt. For this study, 5-week old male Wistar rats were placed on one of two diets for 4 weeks; the control diet contained 14.6% of energy as a peanut/rapeseed oil mix, the intervention diet had 9.7% of energy from peanut/rapeseed oil and 4.9% of energy from fish oil. Prior to termination, rats received an interperitoneal injection of insulin. Western blot analysis showed that the intervention diet resulted in non-significant reductions in insulin-stimulated Akt phosphorylation compared to the control diet.

b) SAPK/JNK

There have not been many studies investigating the effects of dietary oils on SAPK/JNK activity. One study by Todoric *et al*. (2006) involved 6 weeks of high-fat feeding in *db/db* mice. They implemented two high-fat diets (30% of energy from fat): a lard-based diet which was high in SFAs and MUFAs, and an n-3 PUFA diet which contained a mix of safflower oil and EPA/DHA (60% and 40% of fat by weight, respectively). There was also a low-fat control diet (3% of energy from fat). The results of this study showed that, in adipose tissue, SAPK/JNK activation (determined by Western Blot analysis) was similar in the low fat and n-3 groups, and trended ($p<0.1$) towards being significantly higher in the lard group compared to the low-fat control.

Diet-Induced Obese Rat Model

There are a number of different animal models that have traditionally been used to study the effects of obesity and insulin resistance. Among these models is the diet induced obese (DIO) rat. Half of the generic Sprague-Dawley rats will develop diet-induced obesity when placed on a high-fat, high-energy diet and are labelled as obese prone (OP); the other half are obese resistant (OR) because, when placed on the same high-energy diet, they will gain no more weight than rats fed a low-fat, low-energy diet (Levin *et al*., 2003). It is possible to selectively breed the rats and, after three to five generations, OP or OR traits will persist (Reuter, 2004).

Sprague-Dawley OP rats are a more desirable model than transgenic or knockout models because they are more representative of human obesity. OP rats follow a polygenic mode of obesity inheritance which is similar to typical obesity in humans (Levin *et al*., 2003; Reuter, 2004). A disadvantage of this model, though, is that complete development of DM-2 is rare (Reuter, 2004); however, these rats become insulin resistant which is reflected as hyperinsulinemia (Levin *et al*., 1997; Levin *et al*., 2003; Reuter, 2004; Triscari *et al*., 1985) and hyperglycemia (Reuter, 2004). Furthermore, serum TGs increase with an increase in lipolysis (Triscari *et al*., 1985) and resemble what occurs in obesity and insulin resistance in humans. Impairments of parameters of the insulin signalling cascade also occur in this model (Buettner *et al*.,2007).

Summary and Limitations of Published Research

Obesity and DM-2 are health issues of major concern in Canada. Much research has been conducted in these areas to determine changes that occur in specific parameters

at the whole body, tissue, and molecular levels. Studies also indicate that, in various models placed on a high fat diet, the FA composition of the diet is associated with changes in these parameters. General findings are that diets containing SFAs or n-6 PUFAs as the main source of fat are related to detrimental changes in obesity and insulin resistance, MUFAs show conflicting effects (beneficial, negative, or neutral), while VLC n-3 PUFAs are beneficial. Short chain n-3 PUFAs have also been shown to be beneficial in some areas while being unfavourable in others; however, these unfavourable associations are modest compared to diets high in SFAs or n-6 PUFAs and could potentially be explained by an unfavourable ratio of n-6:n-3 PUFAs .

It has been pointed out that the ratio of n-6:n-3 PUFAs in the diet is important when investigating obesity and insulin resistance (Storlein *et al*., 1991). A ratio of less than 10:1 is the current recommendation (Institute of Medicine of the National Academies, 2002) which allows for minimal competition between short chain n-6 and n-3 PUFAs for desaturation and elongation enzymes. As a result, short chain n-3 PUFAs can be adequately converted into EPA and DHA and incorporated into muscle PLs as such, thus reducing the effects of insulin resistance in muscle tissue (Storlein *et al*., 1991).

One of the main limitations of the current body of knowledge is that it lacks sufficient information regarding plant-based sources of n-3 PUFAs. Although the benefits of marine sources of n-3 PUFAs have been demonstrated, these benefits cannot be generalized to all n-3 PUFAs. Additionally, limited research has been conducted with canola oil in the area of obesity and insulin resistance. Canola oil has a unique composition as it is high in MUFAs and ALA and has a favourable n-6:n-3 ratio of approximately 2:1. Only one study reviewed used rapeseed oil (which was assumed to be

analogous to canola oil) and showed moderately detrimental effects (Ikemoto *et al*., 1996). Another study showed beneficial effects of an oil that has relatively high amounts of MUFAs and ALA, however, the oil was created by mixing high-oleic safflower, flaxseed, and corn oils so composition of this oil was not exactly the same as canola oil (Mustad *et al*., 2006).

Other limitations include the types of models, and the study designs, used. Of the studies reviewed, none used the DIO rat model; as mentioned, this model is most representative of the human condition as it develops obesity as a result of multiple gene variations as opposed to commonly used knock-out models. In terms of study design, most studies use a "prevention design" (dietary intervention from baseline) instead of a "treatment design" (dietary intervention after obesity has been established); a treatment design is more appropriate if the findings of a study are to be adapted to obesity treatment strategies in humans.

IX. STUDY RATIONALE

Overall, this study aims to achieve a more complete understanding of the treatment effects of dietary oils on obesity and insulin resistance. First, this study uses a variety of six plant-derived oils for the dietary interventions. This allows for direct comparisons among oils, which is currently difficult to do, as most published studies only compare a few oils at most. Additionally, the use of plant-derived oils will add to the understanding of plant-based n-3 PUFAs.

Second, this study employs a DIO rat model. As mentioned, the DIO rat is a desirable model as it is more representative of human obesity than other commonly used models. Furthermore, this model has not previously been used to investigate the treatment effects of dietary oils on obesity and insulin resistance. Thus, this study provides a novel approach to understanding the possible treatment strategies for inherited obesity.

Last, this study has a "treatment design", whereby obesity will first be established in the animals and then the dietary intervention will be implemented. Although prevention is, agreeably, the best strategy to reduce the prevalence of obesity, an effective treatment strategy is needed for those already living with this condition.

X. HYPOTHESES

1. Diets containing high amounts of ALA (with low n-6:n-3 ratios) will produce beneficial changes in parameters associated with obesity and insulin resistance at whole body and tissue levels; the canola/flax oil diet will be most favourable, followed by the canola oil diet, then the high-oleic canola oil diet. Diets high in SFAs and LA will produce detrimental changes in parameters associated with obesity and insulin resistance.
2. Diets high in ALA will improve the conditions of obesity and insulin resistance by a mechanism involving promotion of normal function of proteins involved in insulin signalling (Akt and SAPK/JNK).

XI. OBJECTIVES

To investigate the hypotheses, the objectives of this study are:

1. To determine the effects of high-fat diets with varying FA compositions on:
    a) Obesity by measuring total body weight accumulation and fat pad/body weight ratios.
    b) Insulin resistance by performing OGTTs and ITTs and by measuring fasting serum insulin, glucose, TGs, and FFAs.
    c) FA composition of muscle PLs.
2. To determine if diets high in ALA improve insulin sensitivity by increasing and/or preventing reduction in phosphorylation of Akt (at sites serine-473 and threonine-308) and reducing and/or preventing increases in phosphorylation of SAPK/JNK (at sites threonine-183 and tyrosine-185) in skeletal muscle.

XII. METHODS

Animals and Diets

a) Diet-induced obesity development phase (weeks 0-12)

One hundred and thirty male Obese-Prone Sprague-Dawley rats were purchased from Charles River Laboratories (St. Constant, PQ). The rats were approximately 7 weeks old at arrival and were acclimatized for 11-16 days. They were housed individually in flat-bottomed polycarbonate cages (containing shavings, cardboard tube, and Nylabone chew toy) and were exposed to a 12 hour light-dark cycle. These conditions were maintained for the duration of the study. After the acclimatization period, the rats were split into two major groups: ninety-six rats were placed on a high saturated fat (lard-based) diet (L); twenty-three rats were placed on a low-fat diet (LF). Twelve rats from each diet group were randomly selected to form a subgroup of 24 rats (see below) to serve as a baseline for the treatment phase. The rats were fed this way for 12 weeks with access to food and water *ad libitum.* The aim of this portion of the study was to develop diet-induced obesity in the lard-fed rats while having the low-fat fed rats as a control. Studies have shown that 12 weeks is sufficient to establish obesity characteristics in the DIO-OP rat model (Levin & Keesey, 1998; Madsen *et al*., 2010).

The diet formulations (Table 3 shows diet formulations, Table 4 shows ingredient details) were a modified version of the American Institute of Nutrition's standardized rodent diet for growth formulation (AIN-93G). The two diets were modified to keep protein content consistent (15% of energy) while altering fat and carbohydrate content. The L diet contained 55% and 30% of energy from fat and carbohydrate, respectively. The LF diet contained 25% and 60% of energy from fat and carbohydrate, respectively.

Although 55% of energy from fat is not recommended for human consumption (Trumbo *et al*., 2002), a dietary fat intake of this level was desired in order to emphasize any potential effects due to the dietary oils. Additionally, the LF diet`s fat content (25% of energy) was chosen in order to avoid a high-carbohydrate content, which may have detrimental effects in obesity and insulin resistance (Volek *et al*., 2004), and to provide a fat content within the acceptable macronutrient distribution range (AMDR) for humans (Trumbo *et al*., 2002). At first, water was added to both the L and LF diets to give them more of a liquid consistency to prevent spillage. However, adding water to the L diet did not change the consistency dramatically and resulted in premature spoilage. After approximately 2 weeks, the L diet was changed to a no water formulation.

Diets were prepared approximately every 2 weeks. To prepare the diets, dry ingredients were weighed out, combined, and stored at -20°C until liquid ingredients were added. All ingredients were mixed together until thoroughly combined. The diets were prepared in 6 kg batches and were stored at -20°C until use when they were stored at 4-6°C.

During this phase, the rats received fresh feed three times per week, body weight measurements were taken bi-weekly, and feed intake was not recorded at this time. At week 8, 10 lard and 10 low-fat fed rats were selected from the aforementioned subgroup (made up of 12 lard and 12 low-fat fed rats) to serve as a baseline for the treatment portion of the study (weeks 12-20). The selection criterion was based on body weights at week 8; the 2 rats with the lowest weights in each of the diet groups were excluded and the remaining rats were used for insulin tolerance tests (week 10) and oral glucose tolerance tests (week 11); all 24 rats were terminated at week 12.

b) Treatment phase (weeks 12-20)

During week 10, all rats not to be terminated at week 12 were evaluated for body weight; those weighing less than 500 grams or greater than 625 grams were determined to be "low gainers" or "outliers", respectively, and were labeled as "alternates". As the DIO rat model develops obesity due to multiple gene variations, it is expected that not all obese-prone rats will actually develop obesity. The selection criteria used here mimics what was used to distinguish between obese-prone and obese-resistant Sprague-Dawley rats (Levin & Keesey, 1998). The remaining rats (labeled as "selected") were randomly assigned to 1 of 6 oil diets (n=10) or kept on lard diet (n=10) for 8 weeks. In two cases, rats weighing less than 500 grams had to be used to maintain 10 rats per group. These two rats were selected using criteria based on a combination of body weight at week 10 (>485 grams) as well as weight gain over 10 weeks (>350 grams). The literature is lacking on what the ideal treatment phase length is for this model. Prevention studies employing rodent models (cited in the literature review) range from 4 to 19 weeks in duration (Ikemoto *et al*., 1996; Okuno *et al*., 1997; Mori *et al*., 1997; Mustad *et al*., 2006; Storlein *et al*., 1991). Therefore, it was assumed that 8 weeks would be sufficient to observe any potential treatment effects of the dietary oils (Figure 2 shows diet groups and study timeline).

The alternate rats were also randomly assigned to oil or lard diets but were excluded from testing [fasting blood collection (week 16), ITT (week 18), and OGTT (week 19)]; they were kept and used to maintain 10 rats per groups if a selected rat became ill and/or died, provided their body weight was not significantly lower than the rest of the group. Table 5 shows use of alternates.

Similar to the lard fed rats, ten of the remaining low-fat fed rats were selected to continue on as a control group while keeping 1 alternate in the group (the lowest gainer).

Oil, lard, and low-fat diets were prepared and stored the same way as during the obesity development phase of the study except that the oil diets were prepared in 10 kg batches and all diets were prepared approximately every 4 weeks. The rats were given fresh food 2 times per week (feed intake was recorded at these times) and body weight measurements were recorded weekly.

Note: A weight-matched group (fed the L diet) was also included for the duration of the treatment phase. This group was to have their feed restricted in order to match their body weights with the lowest weight group and therefore act as a "weight control" group. The weight-matched group did not end up being used as there were no significant differences in body weight among groups at the end of the study (see Results section).

Table 3. Experimental diet formulations[1]

	HC	C	CM	CF	SF	SB	L	LF
Cornstarch	209	209	209	209	209	209	209	347
Maltodextrin	69.4	69.4	69.4	69.4	69.4	69.4	69.4	115.3
Sucrose	100	100	100	100	100	100	100	165.7
Cellulose	63.8	63.8	63.8	63.8	63.8	63.8	63.8	50.2
Casein	186.2	186.2	186.2	186.2	186.2	186.2	186.2	155.5
High oleic canola oil	308.3	0	154.2	0	0	0	0	0
Canola oil	0	308.3	154.2	231.2	0	0	0	0
Flax oil	0	0	0	77.1	0	0	0	0
Safflower oil	0	0	0	0	308.3	0	0	0
Soybean oil	0	0	0	0	0	308.3	28.5	116.5
Lard	0	0	0	0	0	0	279.8	0
AIN-93G- MX[2]	44.6	44.6	44.6	44.6	44.6	44.6	44.6	35
AIN-93-VX[3]	12.7	12.7	12.7	12.7	12.7	12.7	12.7	10
L-Cystine	3	3	3	3	3	3	3	2.3
Choline bitartrate	3.2	3.2	3.2	3.2	3.2	3.2	3.2	2.5
BHT[4]	0.037	0.037	0.037	0.037	0.037	0.037	0.037	0.014
Deionized Water	0	0	0	0	0	0	0	300

[1]Values expressed as g/kg of diet, except low fat diet which is expressed as g/1.3 kg of diet. HC, high-oleic canola; C, conventional canola; CM, HC/C mix; CF, C/flax mix; SF, safflower; SB, soybean; L, lard; LF, low fat.
[2]AIN-93G mineral mix
[3]AIN-93G vitamin mix
[4]2,6-Di-*tert*-butyl-4-methylphenol

Table 4. Ingredient details

Supplier and Location	Ingredient	Other Name/Information
Sigma-Aldrich, St. Louis, MO	2,6-Di-*tert*-butyl-4-methylphenol	BHT
Dyets Inc. - Animal Test Diets for Science and Industry, Bethlehem, PN	AIN-93 VX	Vitamin mix
	AIN-93GMX	Mineral Mix
	Choline Bitartrate	n/a
	Cellulose Fibre, FCC	n/a
	Cornstarch, Food Grade	n/a
	L-Cystine	n/a
	Maltose Dextrin (Fine)	n/a
	Sucrose	n/a
	Teklad Vitamin Free Casein	n/a
Bunge Canada, Oakville, ON	Capri Canola Oil[2]	Contains dimethylpolysilloxane[1]
	Capri Vegetable Oil[3]	Contains dimethylpolysilloxane[1]
	Jubilee Deep Fry Beef Fat[4]	Contains dimethylpolysilloxane[1]
	Nutra-Clear™ High-Stability Canola Oil[5]	Contains dimethylpolysilloxane[1]
Omega Nutrition, Vancouver, BC	Certified Organic Flax Oil[6]	Organic unrefined flax oil
Alnoroil Company, Inc., Valley Stream, NY	Refined High Linoleic Safflower Oil[7]	n/a

[1]Anti-foaming agent
[2]Canola oil
[3]Soybean oil
[4]Lard
[5]High-oleic canola oil
[6]Flax oil
[7]Safflower oil

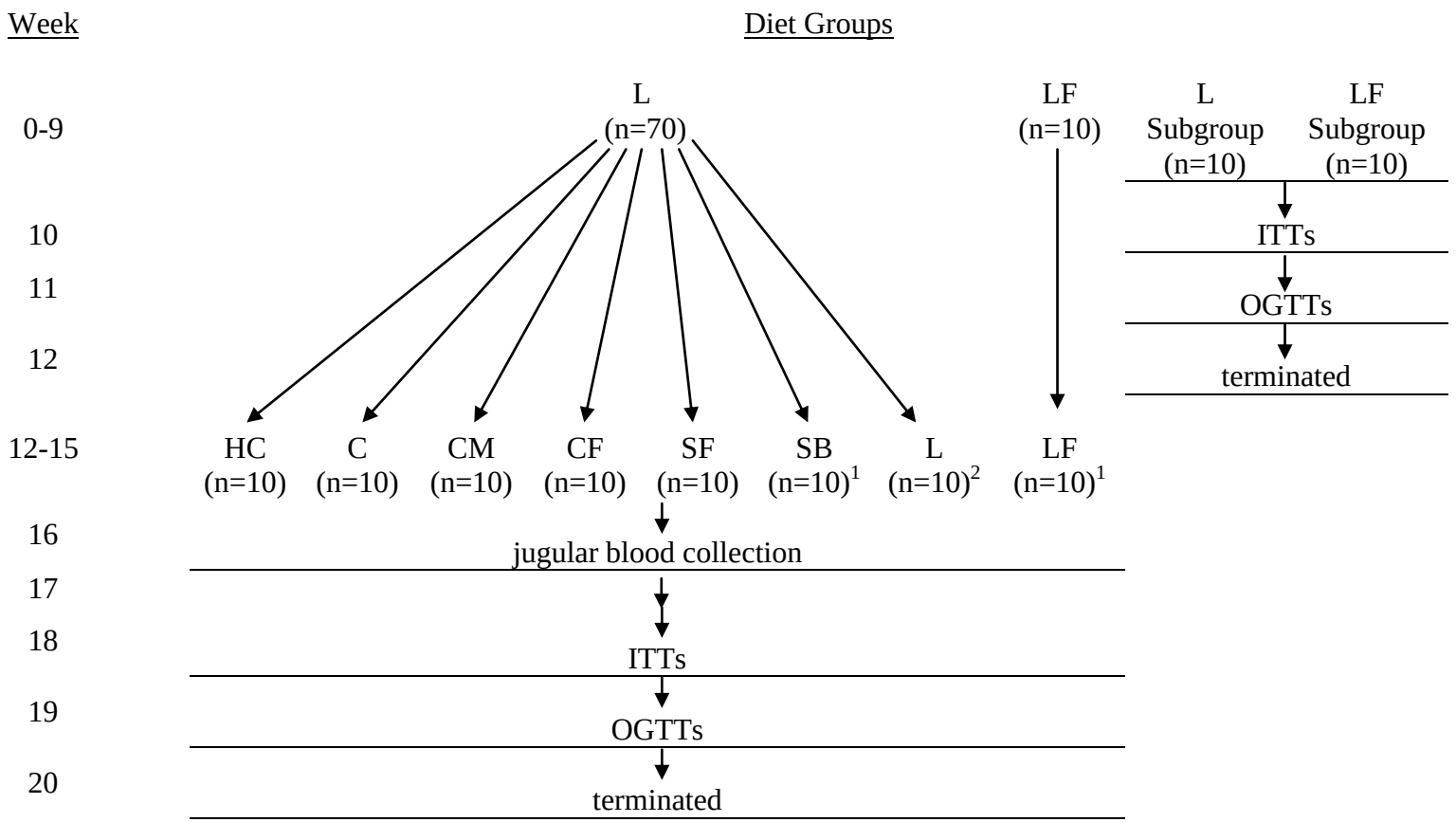


Figure 2. Diet groups and timeline. All groups had 2 alternates unless indicated otherwise. [1]1 alternate. [2]3 alternates. HC, high-oleic canola; C, conventional canola; CM, HC/C mix; CF, C/flax mix; SF, safflower; SB, soybean; L, lard; LF, low fat; ITTs, insulin tolerance tests; OGTTs, oral glucose tolerance tests.

Table 5. Alternate rat use

Selected Rat Number	Alternate Rat Number	Diet Group	Reason For Alternate Use	Week of Alternate Inclusion
115	122	safflower	115 died while having jugular blood collection performed.	17
31	57	canola flax	31 developed a hematoma on right hind leg. Feed intake and weight gain were significantly reduced.	18

Tolerance Tests

a) Insulin tolerance tests

ITTs were performed as a way to assess insulin sensitivity through glucose clearance. In the weeks leading up to ITTs, rats were handled regularly to reduce the potential for stress-related increases in serum glucose. For this procedure, rats are restrained by wrapping them in a towel, a baseline blood glucose measurement is taken, an intraperitoneal injection of insulin is administered, and blood glucose is measured at certain time intervals afterward; animals with larger reductions in blood glucose are more insulin sensitive than those with lower reductions.

Procedure:

At weeks 10 and 18, ITTs were performed. Rats were fasted in clean cages with access to water *ad libitum* for 5 hours. Baseline blood glucose was measured and then a dose of insulin (0.75 U/kg body weight in a mixture of 15 μL insulin/10 mL sterile phosphate buffered saline) was injected into the intraperitoneal space. Blood glucose was measured again at 15, 30, 45, and 60 minutes after the insulin injection. All blood glucose measurements were performed by pricking the tail with a 25 gauge sterile lancet (AlphaTRAK, Abbott Laboratories Inc., Chicago, IL) to obtain a blood droplet and an AlphaTRAK glucometer and test strips were used to determine the blood glucose concentration. Insulin (Novolin®**ge** Human Biosynthetic regular) was from Novo Nordisk Canada Inc. (Toronto, ON, DIN 02024233) and dilution with sterile phosphate buffered saline was performed under sterile conditions in a cell culture hood. Injections were performed with 1 mL Tuberculin Slip Tip syringes (Becton Dickinson, Franklin Lakes, NJ, Ref. # 309602) and Monoject Magellan™ safety needles (Tyco Healthcare,

Mansfield, MA, Cat. # 515-60-17). Upon completion of the final blood glucose measurement, rats were fed 1 mL of a 50% dextrose solution (Baxter Corporation, Mississauga, ON, DIN 02014866) to help recover blood glucose levels and to acclimatize them for OGTTs.

b) Oral glucose tolerance tests

Although the hyperinsulinemic euglycemic clamp method is often considered the gold standard for evaluating insulin sensitivity, it is also more invasive and complicated than a test such as the OGTT (Singh & Saxena, 2010). OGTTs are performed by restraining the rat in a towel, shaving the rat's leg over the saphenous vein, taking a baseline blood sample, administering an oral dose of glucose, and collecting blood at certain time points afterward. The blood collected can be used for measuring serum glucose and insulin concentrations; calculations using these values have been developed to evaluate insulin sensitivity.

The calculation used for this study, which has been validated for OGTT (Allison *et al.*,1995), required determination of area under the curve for glucose (AUC_G) and insulin (AUC_I) by use of the Trapezoidal method (Purves, 1992). More specifically, AUC was calculated by obtaining an average serum concentration of two chronological time points and multiplying by the difference in time points (in minutes) and then all the values were added to obtain the AUC; for example: $([T_0 + T_{15}]/2 \times 15) + ([T_{15} + T_{30}]/2 \times 15) + ([T_{30} + T_{60}]/2 \times 30) = AUC$. A glucose insulin index (AUC_{GI}) (Myllynen *et al.*, 1987), which provides a measure of insulin resistance, was then calculated by multiplying AUC_G by AUC_I.

Procedure:

OGTTs were performed at weeks 11 and 19. Rats were fasted with access to water *ad libitum* in metabolic cages for 5 hours. Baseline blood samples were taken and time of collection completion was recorded. Glucose (Sigma-Aldrich, St Louis, MO, Cat. # G7528-250G) was administered orally (1 g/kg body weight) in a mixture of 0.7 g/mL glucose/double distilled water (ddH_2O) using 1 mL Tuberculin Slip Tip syringes (Becton Dickinson, Franklin Lakes, NJ, Ref. # 309602) and time of completion of glucose consumption was recorded. Blood was collected from the saphenous vein at 15, 30, and 60 minutes after completion of oral glucose load. All blood collections were done by poking the saphenous vein with 22G1 "precision glide" needles (Becton Dickinson, Franklin Lakes, NJ, Ref. # 305155). Blood was collected in CB 300 Z microvette tubes (Sarstedt, Germany, Ref. # 16.440.100); the microvette tubes contained clot activator and held a maximum volume of 300 µL. In total, no blood collection exceeded 1500 µL which is well below the maximum allotted blood draw of 10% of total blood volume every 2 weeks (10% blood volume of a 600 g rat is approximately 3600 µL). Blood was kept on ice and centrifuged (Eppendorf Centrifuge 5804R, Hauppauge, NY) at 2000 rpm (424 g) for 8 minutes at 4°C. If serum did not separate well, samples were stirred and re-centrifuged at 2000 rpm for 3 minutes at 4°C. Serum was collected and placed in 0.5 mL microcentrifuge tubes (Fisher Scientific, Ottawa, ON, Cat. # 05-408-128) and held at -80°C until serum glucose and insulin assays could be performed.

Jugular Blood Collection

Samples of jugular blood were collected at weeks 12 and 16 by animal care staff.

Rats were fasted in metabolic cages with access to water *ad libitum* for 5 hours. Samples (<10% of total blood volume) were collected, under isoflurane inhalation anaesthesia (ABBOTT, Montreal, QC, DIN 02032384), from the jugular vein and placed into 2.0 mL microcentrifuge tubes (Fisher Scientific, Ottawa, ON, Cat. # 02-681-258). The samples were then centrifuged at 2000 rpm for 15 minutes at 4°C and serum was collected. If samples did not separate adequately, they were stirred and re-centrifuged at 2000 rpm for 3 minutes at 4°C. Serum was placed in 0.5 mL microcentrifuge tubes (Fisher Scientific, Ottawa, ON, Cat. # 05-408-128) and stored at -80°C until serum biochemistry analyses could be performed.

Termination and Tissue Collection

At weeks 12 and 20, terminations and tissue collections were performed. Rats were fasted for 12 hours and then were euthanized by carbon dioxide asphyxiation. Body weight and length (nasal to anal length) were recorded. Although not validated as obesity assessment techniques in rodents, body length to weight ratios were used to compare adiposity among groups. To ensure death, the rats were subjected to cervical dislocation. Trunk blood was collected in 15 mL centrifuge tubes (Corning Incorporated, Corning, NY, Cat. # 430052) placed on ice, then centrifuged at 2000 rpm (702g) for 15 minutes at 4°C. Serum was collected, aliquotted into 1.5 mL microcentrifuge tubes (Fisher Scientific, Ottawa, ON, Cat. # 05-664-3), and stored at -80°C until serum biochemistry analyses could be performed. If blood samples did not separate adequately, they were stirred and re-centrifuged at 2000 rpm for 3 minutes at 4°C.

Organs were rapidly dissected, weighed, frozen in liquid nitrogen and stored at

-80°C. Fat pad (peri-renal, epididymal, and mesenteric) to body weight ratios were used as an additional method for determining adiposity. The gastrocnemius muscles were later used to determine phospholipid fatty acid composition and to evaluate phosphorylation of select insulin signalling proteins (see methods below).

Serum Biochemistry

For all serum assays, a microplate reader (FLUOstar Omega, BMG Labtech, Offenburg, Germany) and Omega Control Software (Version 1.0, BMG Labtech, Offenburg, Germany) were used to determine the absorbance of each well of the assay plate. If a dilution was used, the results were multiplied by the dilution factor to obtain the concentration of the parameter of interest. Additionally, the standard curve of each plate had a correlation coefficient equal to or greater than 0.9 and all standards, controls, and samples had a coefficient of variation less than 10%. Finally, each sample fell within the range of the standard curve. If any of these conditions were not met, samples were re-assayed with adjusted dilutions as necessary.

a) Serum glucose

Termination serum and serum collected from OGTTs and jugular blood was analyzed for glucose using a colourimetric assay kit from Genzyme Diagnostics (Charlottetown, PEI, Ref. # 220-32).

Principle:

$$\beta\text{-D-glucose} + O_2 + H_2O \xrightarrow{\text{glucose oxidase}} \text{D-gluconic Acid} + H_20_2$$

$$H_2O_2 + \text{hydroxybenzoate} + \text{4-aminoantipyrine} \xrightarrow{\text{peroxidase}} \text{quinoneimine dye} + H_2O$$

The quinoneimine dye that is produced can be measured, based on its absorbance at 505 nm, and is proportional to the amount of glucose in the sample.

Reagents used:

- Glucose colour reagent: A solution containing (after reconstitution with 100 mL ddH_2O) a buffer (pH 7.25 at 25ºC), 0.25 mmol/L 4-aminoantipyrine, 20 mmol/L p-hydroxybenzoate, >40,000 U/L glucose oxidase (microbial), >200 U/L peroxidase (botanical), and preservatives.
- Glucose calibrator (standard): A solution containing 5 mmol/L glucose and preservatives. This solution was serially diluted to produce 3 additional standards at concentrations of 2.5, 1.25, and 0.625 mmol/L.

Procedure:

Samples were thawed on ice, vortexed, and diluted with ddH_2O (5× dilution for termination serum, 4× dilution for OGTT serum). The quality control (DC-TROL Level 1, Genzyme Diagnostics, Charlottetown, PEI, Ref. # SM-052) was reconstituted with 5 mL of ddH_2O and then diluted in the same manner as the samples. Blank, standards, quality control, and samples were vortexed and 5 μL of each were plated in triplicate on a 96-well plate (NUNC™, Roskilde, Denmark, Cat. # 167008). Two hundred microlitres of the glucose colour reagent was then added to each well. The plate was covered with the plate lid, gently tapped on each side, then incubated for 10 minutes at room temperature. After incubation, the absorbance of the colour in each well was measured at 505 nm using a microplate reader. If the glucose concentration of the quality control did not fall between 4.8-5.8 mmol/L, samples were re-assayed.

b) Serum insulin

Termination serum and serum collected from OGTTs and jugular blood was analyzed for insulin using an ultrasensitive enzyme-linked immunosorbent assay kit from ALPCO Immunoassays (Salem, NH, Cat. # 80-INSRTU-E10).

Reagents and materials used:

- Ultrapure H_2O (Cayman Chemical Company, Ann Arbor, MI, Cat. # 40000)
- Enzyme conjugate buffer
- Enzyme conjugate concentrate
- Mammalian insulin controls (low and high)
- Wash buffer concentrate
- Insulin standards (0.0, 0.15, 0.4, 1.0, 3.0, 5.5 ng/mL)
- 96-well plate coated with horseradish peroxidase enzyme labelled monoclonal antibody
- Substrate
- Stop solution

Procedure:

Samples were thawed on ice, vortexed, and diluted with ultrapure water (4× dilution was used for all samples). All reagents were brought to room temperature prior to use. The mammalian insulin controls were reconstituted with 0.6 mL of ultrapure water. Five microlitres of standards, mammalian insulin controls, and samples were plated in triplicate onto the 96-well plate coated with horseradish peroxidase enzyme labelled monoclonal antibody. Seventy-five microlitres of working strength enzyme conjugate buffer (concentrate diluted 11× with buffer) was added to each well. The plate

was then covered with an adhesive plastic sealer and incubated for 2 hours at room temperature while being shaken at 700-900 rpm on a modified vortex. After incubation, the plate was washed 6 times with working strength wash buffer (wash buffer concentrate diluted 21× with ultrapure water), ensuring no liquid remained in the wells after washing was complete (plate was patted dry on a paper towel). Next, 100 µL of substrate was added to each well and the plate was incubated for 30 minutes at room temperature while being shaken at 700-900 rpm. After the incubation period, 100 µL of stop solution was added to each well and the absorbance of each well was immediately measured at 450 nm using a microplate reader. If the insulin concentration of the mammalian insulin controls did not fall between 0.45-0.88 ng/mL (low) and 2.7-4.79 ng/mL (high), samples were re-assayed. If undiluted samples fell below the range of the standard curve, they were re-assayed with the addition of lower standards (0.02 and 0.05 ng/mL) to the plate.

c) Serum triglycerides

Termination serum and serum from jugular blood were analyzed for TGs with a colourimetric assay kit from Genzyme Diagnostics (Charlottetown, PEI, Cat. # 236-60).

Principle:

$$\text{triglycerides} + H_2O \xrightarrow{\text{lipase}} \text{glycerol} + \text{fatty acids}$$

$$\text{glycerol} + \text{ATP} \xrightarrow{\text{GK, } Mg^{++}} \text{glycerol-1-phosphate} + \text{ADP}$$

$$\text{glycerol-1-phosphate} + O_2 \xrightarrow{\text{GPO}} H_2O_2 + \text{dihydroxyacetone phosphate}$$

$$H_2O_2 + \text{p-chlorophenol} + \text{4-aminoantipyrine} \xrightarrow{\text{peroxidase}} \text{quinoneimine dye} + H_2O$$

GK, glycerol kinase; GPO, glycerol phosphate oxidase. The amount of quinoneimine dye produced can be measured, based on its absorbance at 520 nm, and is proportional to the amount of TGs in the sample.

Reagents used:

- Triglyceride reagent: A buffered solution containing 0.4 mmol/L 4-aminoantipyrine, 2.6 mmol/L adenosine triphosphate, 3.0 mmol/L p-chlorophenol, >2400 U/L glycerol phosphate oxidase (microbial), >1000 U/L lipoprotein lipase (microbial), >540 U/L peroxidise (botanical), >400 U/L glycerol kinase (microbial), stabilizers and preservatives.
- Triglyceride calibrator (standard): A solution containing 2.03 mmol/L TGs. This solution was serially diluted to produce 3 additional standards at concentrations of 1.02, 0.51, and 0.25 mmol/L.

Procedure:

Samples were thawed on ice, vortexed and diluted with ddH_2O (a 4× dilution was used for all samples). The quality control (DC-TROL Level 1, Genzyme Diagnostics, Charlottetown, PEI, Ref. # SM-052) was reconstituted with 5 mL of ddH_2O and diluted in the same manner as the samples. Blank, standards, quality control and samples were then vortexed and 10 μL of each were plated in triplicate onto a 96-well plate (NUNC™, Roskilde, Denmark, Cat. # 167008). Two hundred microlitres of the TG reagent was then added. The plate was covered with the plate lid, tapped on all sides to mix the contents of the wells, then incubated at 37°C for 18 minutes. After the incubation period, all bubbles were popped using a small pipette tip and the absorbance of each well was measured at 520 nm using a microplate reader. If the TG concentration of the quality control did not

fall between 1.90-2.61 mmol/L, samples were re-assayed.

d) Serum free fatty acids

Termination serum FFAs were measured using a colourimetric assay kit from Roche Diagnostics (Penzberg, Germany, Cat. # 11 383 175 001).

Principle:

$$\text{free fatty acids} + \text{CoA} + \text{ATP} \xrightarrow{\text{acyl CS}} \text{acyl-CoA} + \text{AMP} + \text{pyrophosphate}$$

$$\text{acyl-CoA} + O_2 \xrightarrow{\text{ACOD}} \text{enoyl-CoA} + H_2O_2$$

$$H_2O_2 + \text{4-AA} + \text{TBHB} \xrightarrow{\text{POD}} \text{red dye} + 2H_2O + \text{HBr}$$

acyl CS, acyl-CoA synthetase; ACOD, acyl-CoA oxidase; TBHB, 2,4,6-tribromo-3-benzoic acid; POD, peroxidase. The amount of red dye produced can be measured and is proportional to the amount of FFAs in the sample.

Reagents used:

- Reaction mixture A: 1 tablet A (ATP, CoA, acyl-CoA synthetase, peroxidise, ascobate oxidase, 4-aminoantipyrine and stabilizers) dissolved in 11 mL potassium phosphate buffer (pH 7.8).
- Reaction mixture B: 1 tablet B (acyl-CoA oxidase and stabilizers) dissolved in 0.6 mL acyl-CoA oxidase dilution solution and stabilizers.
- Solution C: N-ethyl-maleinimide solution and stabilizers.

Procedure:

Samples were thawed on ice and vortexed. For this assay, no quality control was

provided so one sample was chosen to run on every plate as an internal control. Reaction mixture A and B were prepared at room temperature and tablets were allowed to completely dissolve for at least 10 minutes. Using a multichannel pipette, 200 µL of reaction mixture A was added to each well of a 96-well plate (NUNC™, Roskilde, Denmark, Cat. # 167008). Pipette tips were changed between each use as to avoid excessive bubble build up. Blank, undiluted quality control, and undiluted samples were then vortexed and 10 µL of each were plated in triplicate. The plate was then shaken by hand (keeping plate on countertop and moving it in a circular motion) for 30 seconds, covered with the plate lid, and incubated for 10 minutes at room temperature. After the incubation period, 10 µL of solution C was added to each well using a single pipette and all bubbles were popped (by blowing gently on the plate and/or using a small pipette tip). The plate was mixed for 30 seconds using the shaking feature on the microplate reader and the absorbance of each well was measured at 546 nm. This absorbance was labelled A_1. The plate was then removed from the microplate reader and 10 µL of reaction mixture B was added to each well using a single pipette. The plate was again shaken, uncovered, for 20 seconds by hand, then covered with the plate lid, and allowed to incubate at room temperature for 20 minutes. After the incubation period, all bubbles on the plate were popped, the plate was shaken in the plate reader for 30 seconds and the absorbance was measured at 546 nm. This absorbance was labelled A_2. If the FFA concentration of the quality control did not fall between 0.199 and 0.252 mmol/L, samples were re-assayed.

Serum FFA concentrations were calculated using the following formula:

$$C\ (mmol/L) = \frac{V}{\varepsilon \times d \times v} \times \Delta A$$

Where:

C = concentration of FFA in the sample

V = total well volume in mL (0.230 mL)

v = sample volume in mL (0.010 mL)

d = light path in cm (0.53326 cm)

ε = absorption coefficient at 546 nm [$19.3 \times (1 \text{ x } mmol^{-1} \times cm^{-1})^3$]

ΔA = change in absorbance of the sample minus the change in absorbance of the blank;

$(A_2\text{-}A_1)_{sample} - (A_2\text{-}A_1)_{blank}$

Diet Fatty Acid Analysis

During a random diet-making session, samples of each diet were placed into 10 mL scintillation vials and stored at -20°C until FA analysis could be performed. To analyze the fatty acid composition of the diets, total fat (mainly TGs) was extracted from the diet samples. Then, the FAs were methylated and subjected to gas chromatography (GC) for individual identification and relative concentration quantification. All steps (except for using the centrifuge and oven) were conducted in a fumehood and all solvents used were HPLC grade.

a) Fat extraction from diets

To extract fat from the diets, 2 grams of each sample was weighed out and placed into a 100 mL beaker containing 40 mL of 2:1 chloroform:methanol with 0.01% BHT.

Samples were then homogenized for 30 seconds using a Polytron PT 1600 E homogenizer (homogenizer probe was rinsed with 2:1 chloroform:methanol between each sample). The homogenate was filtered into a 100 mL graduated cylinder through Whatman #1 filter paper and 10 mL of 2:1 chloroform:methanol was used to rinse the filter paper and funnel. The volume of the filtrate was noted and 25% of this volume was added as ddH_2O. The graduated cylinder was stoppered, inverted 5 times to mix the contents, and left to separate overnight. Next, the volume of the bottom (chloroform) layer was noted, the top layer was removed as waste, and a specific amount of the chloroform layer was collected for FA analysis (see calculation below).

b) Diet fatty acid analysis

The amount of the chloroform layer needed for FA analysis was calculated as follows:

2 g (weight of original sample) × 31% (weight of fat in diet) = 0.62 g (or 620 mg) of fat

$$\frac{\text{10 mg (desired amount of fat)}}{\text{620 mg}} \times \text{total volume of chloroform layer} = \text{volume of chloroform layer needed}$$

Note: this calculation was used for high-fat diets. For low-fat diets, 2 grams was multiplied by 9%.

The calculated volume of the chloroform layer was the then placed into an 8 mL test tube and evaporated to dryness under nitrogen in a 35°C water bath. One mL of toluene was then added and the test tube was capped and vortexed for 30 seconds. Next, 1.2 mL of methanolic HCl (Supelco Analytical, Bellefonte, PA, Cat. # 33050-U) was added. The samples were vortexed for another 30 seconds and then placed into an 80°C

oven for 1 hour. Once the samples had cooled (10-15 minutes), 1 mL each of ddH_2O and hexane were added. Samples were then vortexed for 20 seconds and centrifuged for 4 minutes at 2000 rpm (702g). The top (lipid and hexane) layer was placed in a clean 8 mL test tube and 2 mL of ddH_2O was added. Again, the samples were vortexed (20 seconds) and centrifuged (2000 rpm for 4 minutes). Finally, the top (lipid and hexane) layer was placed in GC vials which were then sealed with crimp lids and stored at -20°C until GC could be performed.

FAs, and their relative amounts, were determined using GC. Briefly, the principles of GC are as follows: the GC apparatus consists of an injector, column, and detector; the injector is heated to vaporize the samples which are injected onto the column; different FAs within the sample will travel through the column and reach the detector at different rates; FAs are identified by their retention time. For this study, samples were run on a Varian 450-GC with flame ionization detector (Varian, Lake Forest, CA) and a GC capillary column, 30 m × 0.25 mm diameter and 0.25μm film thickness (Varian Lake Forest, CA, Cat. # CP7420). The temperature program was: 70°C hold × 2 minutes, 180°C at 30°C/minute × 1 minute, 200°C at 10°C/minute × 2 minutes, 220°C at 2°C/minute × 10 minutes, 240°C at 20°C/minute × 5 minutes. Total run time was 36.67 minutes. All samples were run with a 10:1 split ratio.

Muscle Fatty Acid Analysis

To analyze the fatty acid composition of the muscle PLs, three main procedures were performed. First, the total fat was extracted from a portion of the muscle tissue. Second, the fats were separated by thin-layer chromatography (TLC) to obtain the PLs.

Last, the FAs were methylated to liberate the FAs and subjected to gas chromatography (GC) for individual identification and relative concentration quantification. All steps (except for using the centrifuge and oven) were conducted in a fumehood and all solvents used were HPLC grade.

a) Fat extraction from muscle tissue

Fat extraction was based on the Bligh and Dyer method (Bligh & Dyer, 1959). Gastrocnemius muscle samples were removed from storage (at –80°C) and kept on ice. Muscle tissue was weighed (0.5 g), placed in a 50 mL screw-top test tube, and kept on ice until 10 mL of 2:1 chloroform:methanol with 0.01% BHT was added. Samples were then homogenized using a Polytron PT 1600 E homogenizer. Between each sample, the Polytron probe was rinsed with 2:1 chloroform:methanol and fibrous tissue was removed. The homogenate was filtered through Whatman #4 filter paper (150 mm) into 12 mL screw-top test tubes and 2.3 mL of 0.73% sodium chloride (prepared with ddH_2O water) was added. Samples were vortexed and centrifuged (10 minutes at 2000 rpm, 702g) and then the top layer was discarded. The bottom layer was rinsed 2 times with TUP (3:48:47 chloroform:methanol:ddH_2O), removing the top layer after each rinse. The bottom layer was transferred to a clean 8 mL test tube and evaporated to dryness under nitrogen in a 30°C water bath. Two mL of 2:1 chloroform:methanol with 0.01% BHT was then added and samples were stored at –20°C until TLC could be performed.

b) Thin-layer chromatography

PLs were separated from the total fat extract using TLC. The method used for TLC was based on the Gasbarro method (1972). For this, Whatman K6 Silica Gel 60A plates (Maidstone, England, Cat. # 4860-820) were scored to create 5 lanes, heated for 30

minutes at 120°C to activate the plates, and then cooled in a dessicator for 30 minutes. Meanwhile, chromatography paper was used to line a TLC tank and solvent was added (80:20:1 petroleum ether:ethyl ether:acetic acid v/v/v) to the tank.

To prepare the samples, 200 μL of the lipid extract was placed in a 4 mL screw-top test tube and 35 μL of internal standard (1.4 mg/mL 1,2-dipentadecanoyl-sn-slycero-3-phosphocholine; Avanti, Alabaster, AL) was added. The samples were then dried under nitrogen in a 30°C water bath, reconstituted with 50 μl 2:1 chloroform:methanol, and vortexed.

The prepared samples were then spotted (using a syringe) 2 cm from the bottom of the plate in a 4 cm band while being dried with nitrogen. The syringe was rinsed 5 times with 2:1 chloroform:methanol in between samples. The plates were then placed in the TLC tank for 30 minutes, or until the solvent had advanced to approximately 1 cm from the top of the plate. The plate was then removed from the tank, allowed to air dry for 3 minutes, and then sprayed with 0.1% 8-anilio-1-napthalene sulfonic acid. Under UV light, the PL band (located at the origin) was outlined and then scraped onto 4” × 4” weighing paper to be transferred into an 8 mL screw-top test tube. One mL of toluene with 0.01% BHT was added, samples were flushed with nitrogen, and then stored at -20°C until methylation.

c) Methylation

To prepare samples for GC analysis, they first had to be methylated. The method used for methylation was based on the Metcalfe, Schmitz, & Pelka method (1966). For this, 1.2 mL of methanolic HCl (Supelco Analytical, Bellefonte, PA, Cat. # 33050-U)

was added to each sample. Samples were then vortexed and heated to 80°C for 1 hour. Once samples had cooled to room temperature (10-15 minutes), 1 mL of ddH_2O was added. Samples were then vortexed and centrifuged (2000 rpm for 10 minutes). The top layer was transferred to a clean 8 mL screw-top test tube and 1 mL of petroleum ether was added to the bottom layer. Again, samples were vortexed and centrifuged (2000 rpm for 10 minutes). The top layer was added to the previously removed top layer and 2 mL of ddH_2O was added to the combined top layers. Samples were vortexed and centrifuged (2000 rpm for 10 minutes) and the top layer was place in GC conical vials and evaporated to dryness under nitrogen in a 30°C water bath. Hexane (150 µL) was added, vials were sealed with crimp lids, and samples were stored at –20°C until GC analysis could be performed.

d) Gas chromatography

Samples were run on a Varian 450-GC with FID detector (Varian, Lake Forest, CA) and a GC capillary column, 30 m × 0.25 mm diameter and 0.25µm film thickness (Varian Lake Forest, CA, Cat. # CP7420). The temperature program was: 70°C hold × 2 minutes, 180°C at 30°C/minute × 1 minute, 200°C at 10°C/minute × 2 minutes, 220°C at 2°C/minute × 10 minutes, 240°C at 20°C/minute × 5 minutes. Total run time was 36.67 minutes. All samples were run with a 10:1 split ratio.

Western Immunoblot Analysis

In order to determine relative levels of proteins involved in insulin signalling, from gastrocnemius muscle, Western Immunoblotting was conducted. The proteins of interest were Akt and SAPK/JNK. Akt is an intermediate in the insulin signalling cascade

and it is active when phosphorylated at sites serine-473 and threonine-308; a reduction in phosphorylation at either/both of these sites indicates impairment in insulin signalling (Glund & Zierath, 2005). SAPK/JNK is activated when it is phosphorylated at its threonine-183 and tyrosine-185 sites; activated SAPK/JNK results in reduced insulin signalling as it causes feedback inhibition of the cascade by inhibiting insulin receptor substrate activity (Lee *et al*., 2003). Western Immunoblotting was conducted to determine levels of the proteins of interest and the degree of phosphorylation at the specific sites mentioned.

For this procedure, protein is extracted from the sample and then run through sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) to separate individual proteins based on their molecular weight. The proteins that are in the gel matrix are then transferred to a polyvinyldiene difluoride (PVDF) membrane. The membrane is then submerged in bovine serum albumin - tris-buffered saline in tween-20 (BSA-TBST) to block non-specific binding sites and then a primary antibody, which binds specifically to the protein of interest, is added. A secondary antibody (conjugated with horseradish peroxidase) is then added which binds to the primary antibody and produces a chemiluminescent agent via oxidation of luminol. The amount of chemiluminescence produced is directly proportional to the amount of protein and can be quantified through exposure to autoradiographic film.

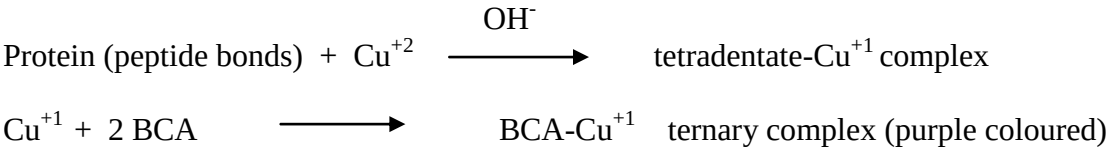
a) Muscle protein extraction

To extract protein from the gastrocnemius muscle, 40 mg of tissue was weighed and placed in a mortar. The tissue was then covered with liquid nitrogen and ground to a powder with a pestle. Once the liquid nitrogen was fully evaporated, 1200 µL of 3×

sample buffer (0.2 M Tris-HCl pH 6.8, 3% SDS, and 30% glycerol) was added and thoroughly mixed with the tissue. The mixture was then allowed to stand for 15 minutes to ensure complete lysation of the cells. After the lysing period, the lysate was placed in a 1.5 mL microcentrifuge tube and centrifuged at 13000 rpm (18000g) for 20 minutes at 4ºC. During this process, the lysate separated into 3 layers: pellet, supernatant, and scum. The protein-containing supernatant was placed in a microcentrifuge tube (the pellet and scum were discarded), and sonicated (Sonic dismembrator, Model 100, Thermo Fisher Scientific Inc., Ottawa, ON) for approximately 10 seconds. Finally, the sonicated samples were stored at -80ºC.

b) Protein assay

Protein assays were conducted to determine the concentration of protein in the protein extracts of the gastrocnemius muscle tissue (all conditions for serum assays apply, see above). These concentrations were needed to load the same amount of protein per lane into the gel (see SDS-PAGE methods below). Protein assays were conducted using a Pierce bicinchoninic acid (BCA) kit (Thermo Scientific, Rockford, IL, Cat. # 23225). The principle behind the protein assay is that when the reagent reacts with the protein, a coloured product is produced. The intensity of the colour can be measured and is directly proportional to the amount of protein in the sample. A schematic of this reaction is provided below:



The amount of ternary complex produced can be measured based on its absorbace at 550 nm and is directly proportional to the amount of protein in the sample.

Reagents Used:

- BCA™ Protein Assay Reagent A (Thermo Scientific, Rockford, IL, Prod.# 23223): contains sodium carbonate, sodium bicarbonate, BCA™ detection reagent, and sodium tartrate in 0.1N sodium hydroxide
- Pierce® BCA Protein Assay Reagent B (Thermo Scientific, Rockford, IL, Prod.# 23224)
- Albumin Standard (Thermo Scientific, Rockford, IL, Prod. # 23209): Contains 2.0 mg/mL bovine serum albumin in a 0.9% aqueous NaCl solution containing sodium azide. The standard was diluted to make 5 additional standards of 1.0, 0.8, 0.6, 0.4, 0.2 mg/mL.

Procedure:

All samples were thawed on ice and diluted with ddH_2O (a 5× dilution was used for all samples). Ten microlitres of blank, protein standards, and samples were plated in triplicate and 200 µL of the working reagent (50 parts reagent A, 1 part reagent B) was added to each well. The plate was then wrapped in parafilm and incubated at 37ºC for 30 minutes. After the incubation period, the plate was read in a microplate reader using a wavelength of 550 nm.

c) Sodium dodecylsulfate polyacrylamide gel electrophoresis

SDS-PAGE is a way to separate proteins based on their molecular weights. Protein samples are heated in SDS-containing buffer and then loaded into the gel. An electric current is applied across the gel to pull the negatively charged proteins through the gel towards the positive electrode. Smaller molecular weight proteins will travel through the gel faster than larger molecular weight proteins. The contents of the gel can

then be transferred to a PVDF membrane which can be used for Western blotting procedures (see below).

A 10% separating gel and 5% stacking gel were used for the SDS-PAGE procedure.

Separating gels:

- 5 mL 20% acrylamide
- 2.25 mL 1.5 M Tris-HCl pH 8.8
- 2.65 mL ddH_2O
- 100 µL 10% ammonium persulfate
- 100 µL 10% SDS
- 8 µL N,N,N',N'-Tetramethylethylenediamine (TEMED) (MP Biomedicals LLC, Solon OH, Cat. # 805615,)

Stacking gels:

- 1 mL 20% acrylamide
- 1 mL 0.5 M Tris-HCl pH 6.8
- 1 mL ddH_2O
- 40 µL 10% ammonium persulfate
- 40 µL 10% SDS
- 10 µL TEMED

Other reagents used:

- H_2O-saturated butanol
- 10% bromophenol blue (BPB) (w/v)
- 2-mercaptoethanol (β-ME) (14.16 M)

- 5× running buffer (200 mL 10% SDS, 288 g glycine, 60.6 g tris-base, ddH_2O to yield a final volume of 4 L)
- 5× transfer buffer (75.75 g Tris-base, 242.5 g glycine, ddH_2O to yield a final volume of 4 L)
- 5× Tris-buffered saline in Tween-20 (TBST) (600 mL 5 M NaCl, 40 mL 1 M tris-HCl pH 7.4, 10 mL Tween-20, ddH_2O to yield a final volume of 4 L)
- methanol

Procedure:

To prepare the separating gel, two glass plates were cleaned with ddH_2O and separated with 1.0 mm spacers. The plates were held together with sandwich clamps and placed in a casting stand. The separating gel mixture was pipetted in between the glass plates and H_2O-saturated butanol was pipetted on top to eliminate bubbles, keep the gel moist while polymerization, and ensure a level gel surface. The separating gel was allowed to polymerize for 1 hour. After polymerization, the H_2O-saturated butanol was rinsed off with ddH_2O and excess ddH_2O was wicked away with a paper towel. The stacking gel was then prepared and pipetted in between the glass plates and a 15-well comb was inserted for the duration of polymerization. The stacking gel was allowed to polymerize for 15 minutes.

Gastrocnemius muscle protein samples were prepared by placing 10 µg of sample in a microcentrifuge tube (see below for sample calculation). Generally, volumes of protein sample were small so 5 µl of 3× sample buffer was added to each sample to aid in loading the samples into the gel. A 1:1 mixture of 10% BPB:β-ME (v/v) was added to each sample in an amount of 10% of sample (i.e. 10 µl sample would have 1 µl 10%

BPB/β-ME added). Samples were then placed in a 90ºC hot water bath for 3 minutes to denature the proteins.

Volume of protein sample calculation:

$$\frac{\text{10 ug of protein (to load into gel)}}{\text{protein concentration (from protein assay) in µg/µl}} = \text{volume of protein sample needed (µl)}$$

Before protein samples could be loaded into the gel, well combs were removed and the sandwich clamp assembly was transferred to an electrophoresis apparatus and placed in a tank. The space in the middle of the tank was filled with working strength running buffer (100 mL 5× running buffer diluted with 400 mL ddH_2O). Samples were loaded into the wells using a glass syringe; the syringe was rinsed with running buffer three times in between each sample. In each gel, a molecular weight marker was loaded into the first well and a standard sample was loaded into the second well. The standard sample was a sample from the lard group which was run on every gel to adjust for intensity differences due to the nature of the Western blot procedure. Once samples were loaded, the remaining running buffer was poured into the tank and the electrophoresis apparatus was connected to a power supply. The power pack was set to 20 mA per gel (2 gels could be run per tank) and allowed to run until the BPB dye front was at the bottom of the gel (approximately 1 hour).

After the run was complete, the gel was transferred to a PVDF membrane. For this procedure, the PVDF membrane was dipped in methanol and then allowed to equilibrate in the working strength transfer buffer (200 mL methanol, 160 mL 5× transfer buffer, ddH_2O to yield a final volume of 1 L) for 5 minutes. In the meantime, the glass plates surrounding the gel were pried apart and the stacking gel was discarded. The gel

was transferred onto blotting paper, covered with the PVDF membrane (ensuring all bubbles were pushed out) and covered with another blotting paper. This package was then placed between two fibre boards and secured into a gel transfer cassette (all materials used were soaked in transfer buffer prior to use). The cassette was clamped shut and placed into the transfer apparatus with the negative side of the cassette facing the negative side of the apparatus. The transfer apparatus was then placed in a tank with transfer buffer, an ice pack, and a stirring rod. The tank was then placed on a stir plate and the power supply was connected to the electrodes of the transfer apparatus. A constant voltage of 100 V was applied to the apparatus for 1 hour. Upon completion of the transfer, the cassette package was disassembled and the PVDF membrane was placed in 1× TBST (1 part 5× TBST, 4 parts ddH_2O) at 4ºC for storage.

d) Western immunoblotting

In order to indentify the protein of interest, Western blot analyses were performed. For this process a primary antibody, specific to the protein of interest, is applied. A secondary antibody conjugated to horseradish peroxidase (HRP) is then added which will bind to the primary antibody. The HRP will catalyze a reaction that results in the emission of light due to production of a chemiluminescent agent. The amount of light produced is directly proportional to the amount of protein present and can be observed as black bands on audioradiographic film.

Reagents and materials used:

- 3% BSA in TBST
- 1× TBST
- 1% BSA in TBST

- Primary antibody
- Secondary antibody conjugated with horseradish peroxidase
- Lumigen PS-3 detection reagent (Amersham™ ECL Plus Western Blotting Detection System, GE Healthcare, Buckinghamshire, UK, Cat. # RPN2132)
- Autoradiographic film (CL-XPosure™ Clear Blue X-Ray Film, Thermo Scientific, Rockford, IL, Cat. # 34091)

Procedure:

To begin, the PVDF membrane was placed in a blotting box, covered with 10 mL of 3% BSA in TBST, and placed on an orbital shaker for 1 hour. After this incubation period, the 3% BSA was discarded and the primary antibody (see Table 6) was added (10 µl of antibody in 10 mL 3% BSA in TBST). The membrane was placed on an orbital shaker overnight at 4°C. Primary antibodies were then placed in 15 mL plastic tubes and frozen at -18ºC for future use. The membranes were washed 4 times for 5 minutes each with 1× TBST. The secondary antibody (see Table 6) was added (1 µL antibody in 10 mL 1% BSA in TBST) for 1 hour while shaking. Again, the membranes were washed as described above. The membranes were then dipped in Lumigen PS-3 detection reagent, placed between two acetate sheets, and put into an autoradiography cassette. In darkness (red light), the autoradiographic film was placed on top of the acetate sheet and the cassette was closed for the duration of the exposure. Exposure times varied as shown in Table 6.

To quantify the intensity of the bands, a densitometer (trace analysis with a GS 800 Imaging Densitometer, Bio-Rad Laboratories, Hercules, CA) and Quantity One

software (Version 4.5.0, Bio-Rad Laboratories, Hercules, CA) were used. Because several different membranes were probed for the same protein, all bands had to be standardized to account for differences in exposure intensity. To do this, the trace quantity (TQ) of each protein of interest was divided by the TQ of the intensity control on the same membrane. Then, to determine degree of protein phosphorylation, the TQs of the phosphorylated protein bands were divided by the TQs of the non-phosphorylated protein bands (for example, pAkt-T308/Akt, where both pAkt-T308 and Akt were standardized as mentioned previously). Likewise, to determine protein levels, the TQs of the non-phosphorylated proteins bands were divided by the TQs of the loading control bands (for example, Akt/β-Tubulin, where both Akt and β-Tubulin were standardized as mentioned previously).

Table 6. Antibody information

Primary	Secondary	Application	Molecular Weight (kDa)	Exposure Time	Catalogue number	Company
Akt	Rabbit	Western Blot	60	7 minutes	9272	Cell Signalling
pAkt Thr^{308}	Rabbit	Western Blot	60	2.5 minutes	9275	Cell Signalling
pAkt Ser^{473}	Rabbit	Western Blot	60	3 minutes	9271	Cell Signalling
SAPK/JNK	Rabbit	Western Blot	46/54	5 minutes	9252	Cell Signalling
pSAPK/JNK Thr^{183}/Tyr^{185}	Rabbit	Western Blot	46/54	3 minutes	9251	Cell Signalling
β-Tubulin	Rabbit	Western Blot	55	3 seconds	2146	Cell Signalling

Thr^{308}, phosphorylated at threonine-308; Ser^{473}, phosphorylated at serine-473; Thr^{183}, phosphorylated at threonine-185; Tyr^{185}, phosphorylated tyrosine-185.

e) Stripping membranes

Membranes could be used for identification of more than one protein, however, the previously used antibodies needed to be stripped off of the membrane.

Reagents used:

- Stripping buffer (400 mL 10% SDS, 62.5 mL 0.5 M Tris-HCl pH 6.8, ddH_2O to yield a final volume of 500 mL)
- β-ME
- 1× TBST

Procedure:

Stripping solution was prepared by combining 25 mL of stripping buffer with 200 µL of β-ME in a plastic sealable container. Up to 3 membranes were placed in the stripping solution at one time. The container was sealed and placed in a secondary sealed container to minimize the odour of the β-ME. The membranes were then placed on an orbital shaker overnight. The stripping solution was discarded and membranes were placed in a clean container, rinsed with 1× TBST in 5 minute intervals until the odour of the β-ME was undetectable, and then stored in 1× TBST at 4°C.

Statistical Analysis

Time course data was analyzed by using repeated measures analysis of variance (ANOVA). Values measured at a single time point were analyzed using ANOVA followed by Duncan's multiple range test. Non-normal and/or non-homogeneous data (as determined by the Shapiro-Wilk test and Levene's test, respectively) were log transformed prior to ANOVA or subjected to Wilcoxon non-parametric testing followed

by least significant differences post hoc testing. Correlation statistics were conducted using Pearson's Correlation. A p-value (or chi-square value in Wilcoxon) <0.05 was considered statistically significant. Statistical analysis software (SAS Version 9.1.3, SAS Institute Inc., Cary, NC) was used for all analyses.

XIII. RESULTS

From this point forward the dietary groups, terminated at week 20, are abbreviated as follows: HC, high-oleic canola; C, conventional canola; CM, HC/C mix; CF, C/flax mix; SF, safflower; SB; soybean; L, lard; LF, low fat. In some instances, rats terminated at week 12 are compared to those terminated at week 20; abbreviations for these cases are: L-12, lard terminated at week 12; L-20, lard terminated at week 20; LF-12, low fat terminated at week 12; LF-20, low fat terminated at week 20. Therefore, L/L-20 and LF/LF-20 represent the same samples, respectively.

Diet Fatty Acid Composition

All diets showed similar FA profiles compared to those stated in the literature (Table 7). In general, the HC, followed by the CM, diets had the highest amount of MUFAs; the C diet was high in MUFAs but also contained a moderate amount of ALA. The CF diet had the lowest amount of MUFAs out of the canola-based diets (HC, C, CM, and CF) but had the highest ALA out of any diet. The SF diet had the highest amount of LA, followed by the SB and LF diets; the SB and LF diets shared nearly identical FA compositions, which was expected, since the LF diet used SB oil as the source of fat. The SF diet also had the lowest ALA content, while the SB and LF diets had moderate amounts of ALA. The L diet had the highest amount of SFAs, low amounts of LA and ALA, and moderate amounts of MUFAs.

Table 7. Diet fatty acid composition

Fatty Acid	HC	C	CM	CF	SF	SB	L	LF
Total SFA	7	8	8	8	11	16	49	17
C16:0	4	4	4	4	7	10	24	10
C18:0	2	2	2	2	2	4	20	4
Total MUFA	76	65	71	54	16	22	42	21
C18:1	71	60	66	50	15	20	37	19
Total PUFA	16	27	22	38	73	62	9	62
LA	15	18	16	18	73	53	8	52
ALA	2	8	5	20	0.2	9	1	9
LA/ALA	8	2	3	1	464	6	6	6
Total n-6	15	19	17	18	73	53	8	53
Total n-3	2	8	5	20	0.2	9	1	9
n-6/n-3	8	2	3	1	464	6	7	6

All data expressed as grams/100 grams fatty acids (n=1 sample per diet). HC, high-oleic canola; C, conventional canola; CM, HC/C mix; CF, C/flax mix; SF, safflower; SB; soybean; L, lard; LF, low fat.

Feed Intake, Body Weight, Organ Weights

a) Feed intake

Feed intake was only measured during the treatment phase of the study. No differences were seen in weekly feed intake, total feed intake, or feed efficiency ratio (Table 8). Feed efficiency ratio was calculated by dividing weight gain (in grams) by total feed intake (in grams).

b) Body weight

As expected, the L-12 and LF-12 groups had significantly lower final body weights than their week 20 counterparts (L-20 and LF-20, respectively), however, the two groups did not differ from each other at the same time point (Table 9). Additionally, the weight gain in these groups did not differ during weeks 0-12 or weeks 12-20 (Table 9).

No differences were seen in weekly body weights (Table 10). Interestingly, differences were seen in body weight gain during the treatment portion of the study; the LF group had the highest amount of weight gain while the HC group had the lowest amount of weight gain (Figure 3).

c) Body weight to length ratio

Although not validated as a measure of obesity in rats, a body weight to length ratio was calculated as a surrogate measure of BMI. As expected, the L and LF groups showed significant increases in body weight to length ratios over the course of the study (Figure 4), however, there were no differences among treatment groups (Figure 5).

Table 8. Weekly feed intake, total feed intake, feed efficiency ratio - weeks 12-20

	HC	C	CM	CF	SF	SB	L	LF[1]
Weekly feed intake[2]								
Week 12	162 ± 22	162 ± 15	167 ± 16	149 ± 13	159 ± 12	149 ± 12	155 ± 11	170 ± 10
Week 13	185 ± 18	203 ± 17	197 ± 15	190 ± 13	186 ± 11	200 ± 14	196 ± 17	192 ± 13
Week 14	168 ± 13	191 ± 13	190 ± 12	176 ± 7	170 ± 9	166 ± 10	206 ± 15	173 ± 6
Week 15	187 ± 16	187 ± 11	189 ± 10	176 ± 6	185 ± 10	177 ± 12	200 ± 13	180 ± 10
Week 16	164 ± 6	177 ± 7	181 ± 6	166 ± 9	157 ± 13	165 ± 9	168 ± 17	172 ± 9
Week 17	187 ± 22	200 ± 18	212 ± 23	187 ± 20	195 ± 20	189 ± 20	175 ± 18	177 ± 13
Week 18	151 ± 7	151 ± 11	171 ± 11	151 ± 7	150 ± 8	153 ± 10	157 ± 10	165 ± 9
Week 19	137 ± 5	136 ± 8	151 ± 13	136 ± 4	150 ± 11	133 ± 9	144 ± 9	145 ± 8
Total feed intake[2]	1348 ± 86	1414 ± 76	1458 ± 74	1334 ± 53	1352 ± 53	1340 ± 74	1404 ± 79	1380 ± 64
Feed efficiency ratio[3]	26.2 ± 2.3	20.0 ± 1.8	18.3 ± 1.4	18.2 ± 1.4	20.2 ± 2.7	18.9 ± 1.1	17.4 ± 1.9	15.0 ± 1.4

All data expressed as mean ± SEM (n=10 rats/group). An absence of superscripts indicates no significant differences at the same time point. See Table 7 for abbreviations.

[1]LF values adjusted for water content. Water was added to the diet to provide a paste-like consistency; values represent weight of feed ingredients only.

[2]data expressed in grams.

[3]calculated by dividing total feed intake in grams by total weight gain in grams.

Table 9. Body weight - week 12 vs. 20

		L-12	L-20	LF-12	LF-20
Final body weight		543 ± 12[b]	655 ± 22[a]	552 ±13[b]	633 ± 20[a]
Weight gain	Weeks 0-12	361 ± 9	390 ± 9	367 ± 12	368 ± 9
	Weeks 12-20	-	79.5 ± 11.3	-	88.6 ± 16.3

All data expressed as mean ± SEM (n=10 rats/group) in grams. Superscripts indicate significant differences ($p<0.05$) by Duncan's multiple range test. L-12, lard terminated at week 12; L-20, lard terminated at week 20; LF-12, low fat terminated at week 12; LF-20, low fat terminated at week 20.

Table 10. Weekly body weight - weeks 12-20

Week	HC	C	CM	CF	SF	SB	L	LF
12	587 ± 14	570 ± 11	581 ± 9	554 ± 8	572 ± 15	569 ± 8	576 ± 14	555 ± 11
13	597 ± 16	591 ± 11	607 ± 10	568 ± 11	589 ± 16	586 ± 9	596 ± 16	578 ± 11
14	609 ± 17	608 ± 12	628 ± 9	586 ± 12	608 ± 16	606 ± 9	615 ± 17	594 ± 12
15	619 ± 18	623 ± 13	644 ± 10	603 ± 13	623 ± 16	622 ± 9	628 ± 17	607 ± 13
16	634 ± 19	637 ± 14	656 ± 10	617 ± 14	638 ± 17	632 ± 9	646 ± 18	620 ± 15
17	643 ± 19	648 ± 14	667 ± 9.6	628 ± 16	646 ± 15	642 ± 9	650 ± 20	628 ± 16
18	653 ± 20	657 ± 15	676 ± 11	636 ± 14	655 ± 15	654 ± 10	662 ± 21	641 ± 18
19	655 ± 20	660 ± 14	680 ± 12	642 ± 14	660 ± 14	660 ± 10	669 ± 21	647 ± 19
20[1]	639 ± 19	639 ± 14	663 ± 11	624 ± 13	647 ± 14	643 ± 10	655 ± 22	643± 20

All data expressed as mean ± SEM (n=9 or 10 rats/group) in grams. An absence of superscripts indicates no significant differences at the same time point. See Table 7 for abbreviations.

[1]Week 20 weights were measured after termination (after a 12 hour fast).

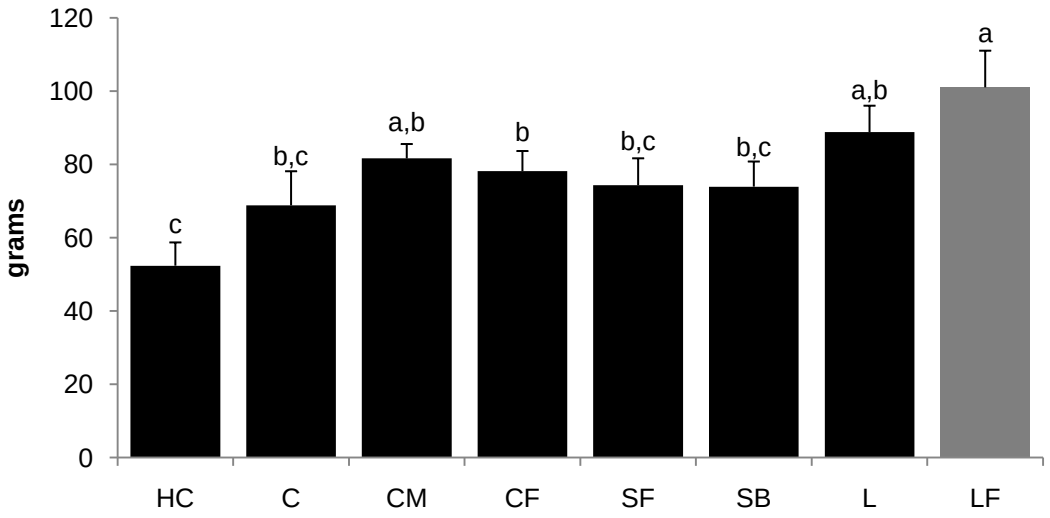


Figure 3. Weight gain - weeks 12 to 20. Data expressed as mean ± SEM (n=9 or 10 rats/group). Different superscripts indicate significant differences ($p<0.05$) by Duncan's multiple range test. See Table 7 for abbreviations. See Appendix 1 for tabulated values.

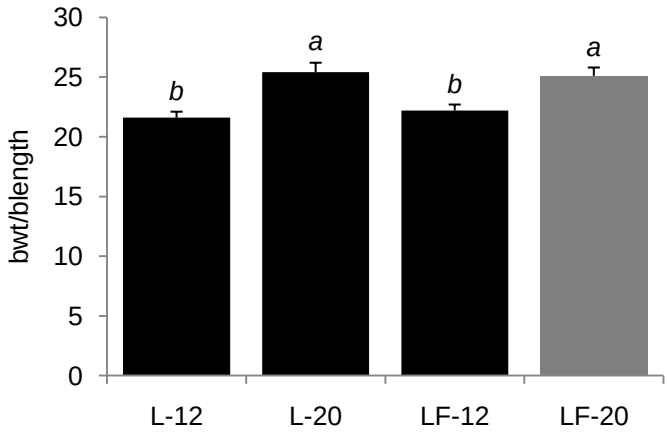


Figure 4. Body weight to length ratio - week 12 vs. 20. Data expressed as mean ± SEM (n=10 rats/group). Different superscripts indicate significant differences ($p<0.05$) by Duncan's multiple range test. See Table 9 for abbreviations; bwt, body weight; blength, body length.

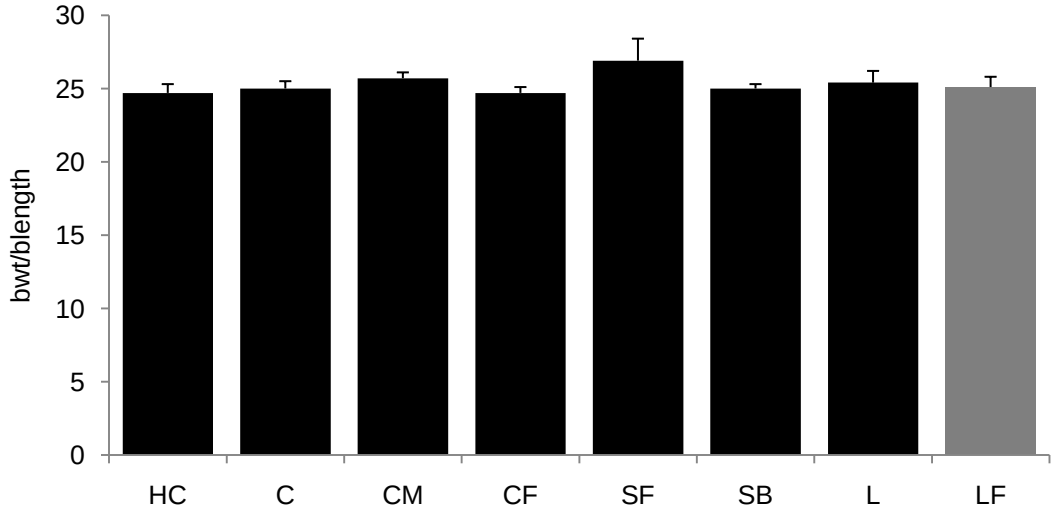


Figure 5. Body weight to length ratio - week 20. Data expressed as mean ± SEM (n=9 or 10 rats/group). An absence of superscripts indicated no significant differences. See Table 7 and Figure 4 for abbreviations.

d) Adipose weight

All adipose weights are expressed as g/100 g body weight in order to standardize values and control for body weight; tabulated values and raw weights are listed in Appendix 2.

Total visceral adipose weight was significantly higher in the L-20 and LF-20 groups compared to the L-12 and LF-12 groups, respectively (Figure 6). Individual fat pad masses followed a similar pattern, except for epididymal adipose tissue which was not significantly different between the different time points (Figures 8, 10, and 12).

At the end of the study, the LF group had less total visceral adipose tissue than the HC, C, CM, and SF groups (Figure 7). Likewise, the LF group had less epididymal adipose tissue compared to all groups except the L group (Figure 9). Additionally, the L group had less epididymal adipose tissue than the C and SF groups (Figure 9). Peri-renal adipose tissue weight was lower in the LF group compared to all other groups (Figure 11) and mesenteric adipose tissue did not differ among groups (Figure 13).

e) Gastrocnemius muscle weight

All gastrocnemius muscle weights are expressed as g/100 g body weight in order to standardize values and control for body weight; tabulated values and raw weights are listed in Appendix 3.

Gastrocnemius muscle weight was lower in the L-20 and LF-20 groups compared to the L-12 and LF-12 groups, respectively (Figure 14). No differences in gastrocnemius muscle weight were observed among groups at the end of the treatment phase (Figure 15).

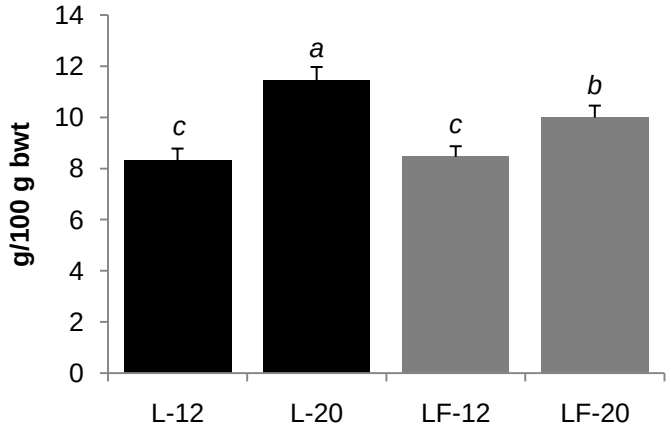


Figure 6. Visceral adipose tissue weight - week 12 vs. 20. Data expressed as mean ± SEM (n=10 rats/group). Different superscripts indicate significant differences ($p<0.05$) by Duncan's multiple range test. See Table 9 for abbreviations; g, grams; bwt, body weight.

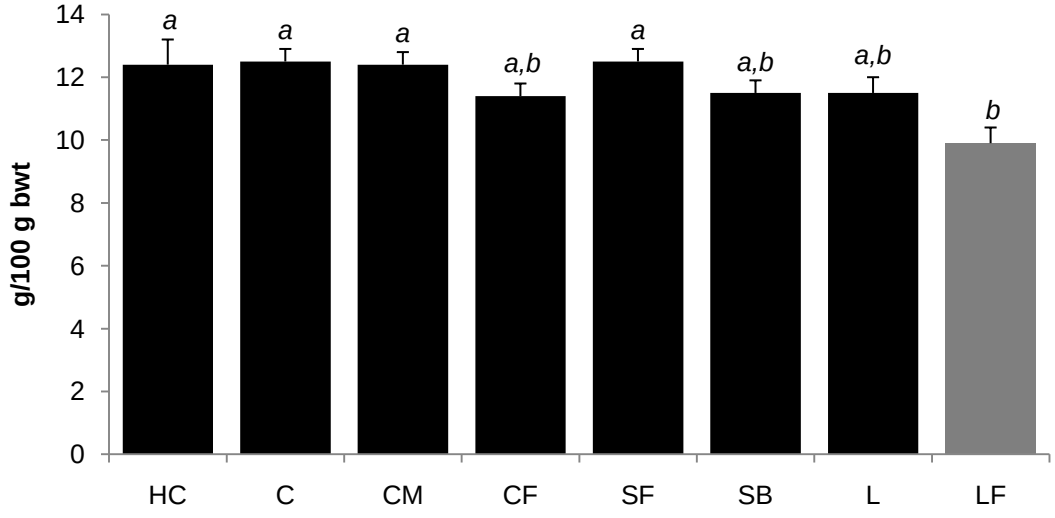


Figure 7. Visceral adipose tissue weight - week 20. Data expressed as mean ± SEM (n=9 or 10 rats/group). Different superscripts indicate significant differences ($p<0.05$) by Duncan's multiple range test. See Table 7 and Figure 6 for abbreviations.

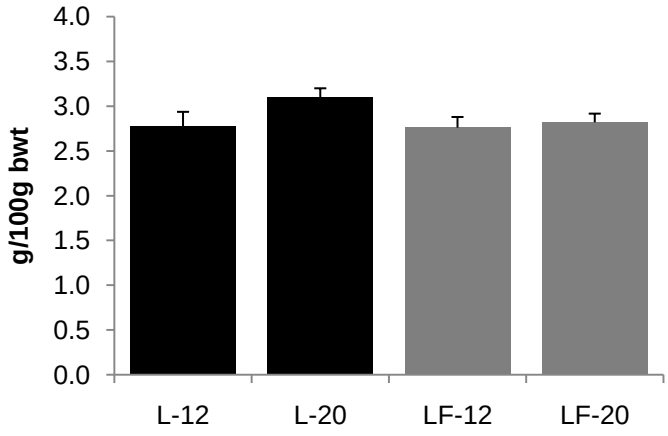


Figure 8. Epididymal adipose tissue weight - week 12 vs. 20. Data expressed as mean ± SEM (n=10 rats/group). An absence of superscripts indicates no significant differences. See Table 9 and Figure 6 for abbreviations.

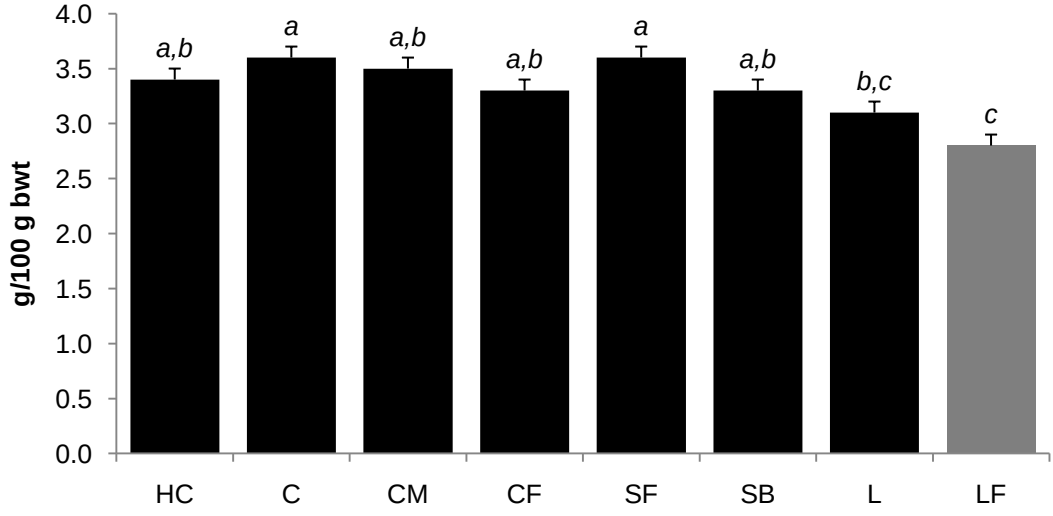


Figure 9. Epididymal adipose tissue weight - week 20. Data expressed as mean ± SEM (n=10 rats/group). Different superscripts indicate significant differences ($p<0.05$) by Duncan's multiple range test. See Table 7 and Figure 6 for abbreviations.

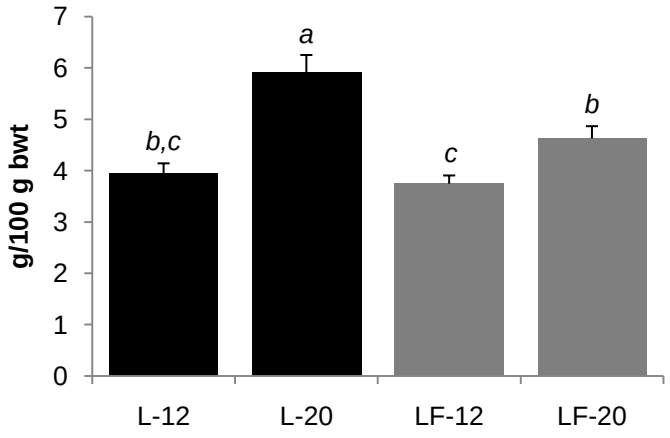


Figure 10. Peri-renal adipose tissue weight - week 12 vs. 20. Data expressed as mean ± SEM (n=10 rats/group). Different superscripts indicate significant differences ($p<0.05$) by Duncan's multiple range test. Data were log transformed prior to ANOVA. See Table 9 and Figure 6 for abbreviations.

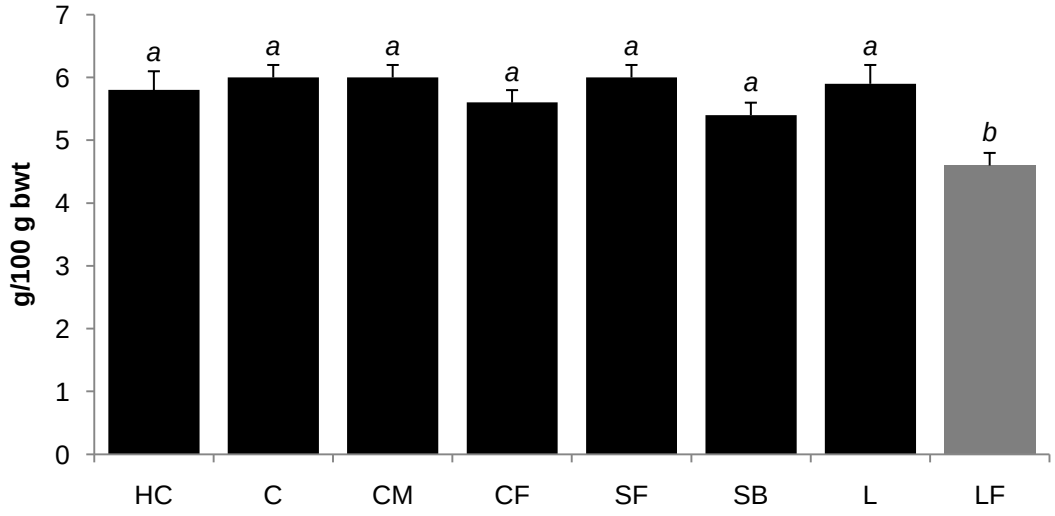


Figure 11. Peri-renal adipose tissue weight - week 20. Data expressed as mean ± SEM (n=9 or 10 rats/group). Different superscripts indicate significant differences ($p<0.05$) by Duncan's multiple range test. See Table 7 and Figure 6 for abbreviations.

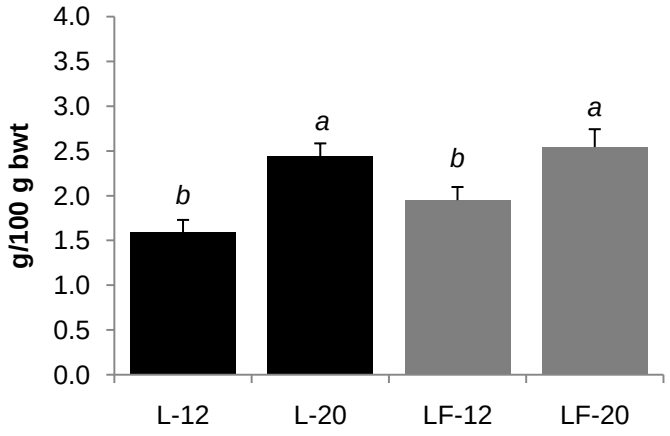


Figure 12. Mesenteric adipose tissue weight - week 12 vs. 20. Data expressed as mean ± SEM (n=10 rats/group). Different superscripts indicate significant differences ($p<0.05$) by Duncan's multiple range test. Data were log transformed prior to ANOVA. See Table 9 and Figure 6 for abbreviations.

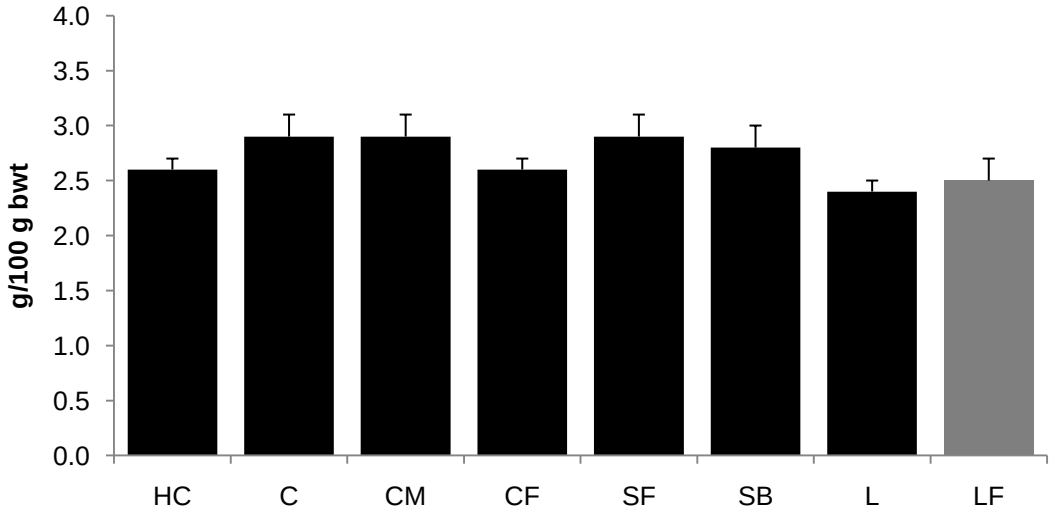


Figure 13. Mesenteric adipose tissue weight - week 20. Data expressed as mean ± SEM (n=9 or 10 rats/group). An absence of superscripts indicates no significant differences. See Table 7 and Figure 6 for abbreviations.

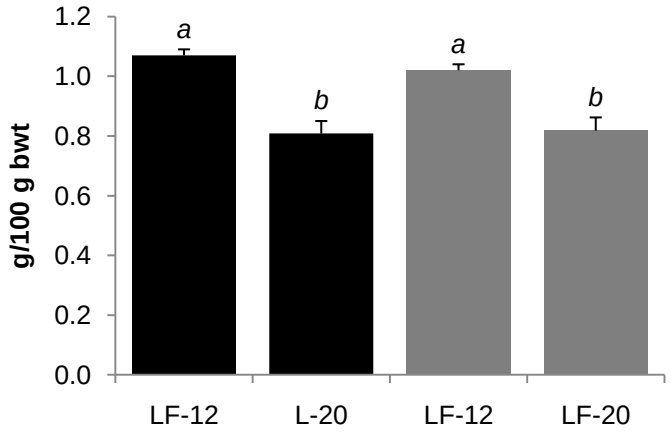


Figure 14. Gastrocnemius muscle mass - week 12 vs. 20. Data expressed as mean ± SEM (n=9 or 10 rats per group). Different superscripts indicate significant differences ($p<0.05$) by Duncan's multiple range test. Data were log transformed prior to ANOVA. See Table 9 and Figure 6 for abbreviations.

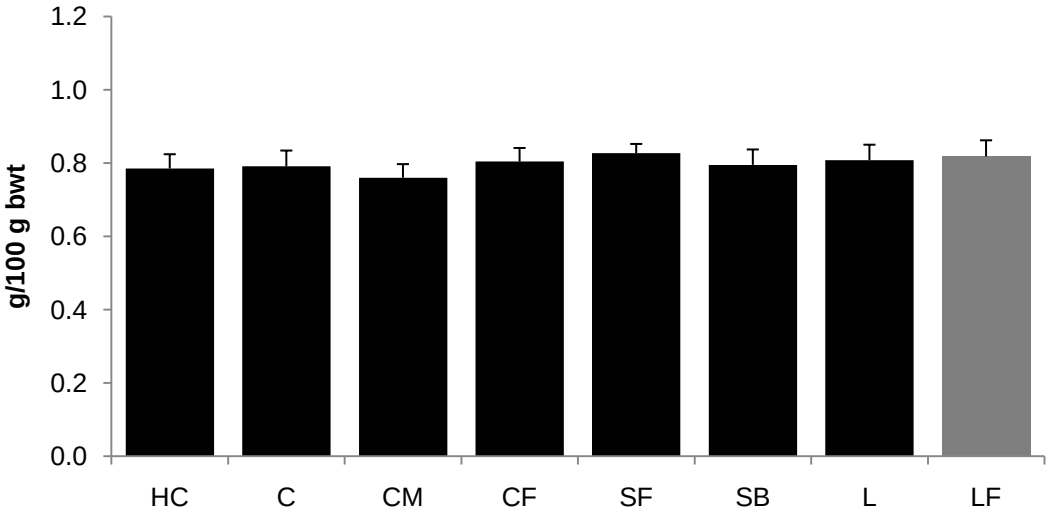


Figure 15. Gastrocnemius muscle mass - week 20. Data expressed as mean ± SEM (n=9 or 10 rats per group). An absence of superscripts indicates no significant differences. See Table 7 and Figure 6 for abbreviations.

Insulin Resistance

Insulin resistance was evaluated using a variety of methods. Fasting serum glucose and insulin were measured at termination as well as at the beginning and middle of the treatment phase; a homeostatic assessment model for insulin resistance (HOMA-IR) score was also calculated for each time point.

a) Glycemia and insulinemia

Fasting serum glucose and insulin concentrations, alone, provide little information regarding the degree of insulin resistance present. Therefore, a homeostatic assessment model for insulin resistance (HOMA-IR) score was also calculated (serum glucose concentration [mmol/L] × serum insulin concentration [μU/mL] / 22.5). This score has been shown to correlate with hyperinsulinemic euglycemic clamp values (Matthews *et al*., 1985).

Fasting serum glucose decreased over the course of the study in the LF, but not the L, groups (Figure 16 a); serum insulin (Figure 17 a) and HOMA-IR (Figure 18 a) did not differ over the course of the study in these groups.

Fasting serum glucose was not significantly different among treatment groups at week 12 or 16, but, at the end of the study, the C group had significantly lower serum glucose than all other groups except the SB group (Figure 16 b, c, and d). At week 12, none of the treatment groups had different serum insulin compared to the L group, however, the HC and C groups had lower serum insulin than the SB group (Figure 17 b). There were no differences in serum insulin at week 16, but, at week 20, the C, CF, and SF groups had lower serum insulin than the L group (Figure 17 c and d). HOMA-IR scores were not different among groups at week 12 and 16 (Figure 18 b and c). At the end

of the study, the C, CF, and SF groups had lower HOMA-IR scores than the L group (Figure 18 d). All values are tabulated in Appendix 4.

b) Insulin tolerance tests

No differences were seen in the ITTs over the course of the study in the L and LF groups (Table 11). Likewise, there were no significant differences in ITTs among treatment groups at the end of the study (Table 12).

c) Oral glucose tolerance tests

Similar to ITTs, no differences were seen in OGTTs over the course of the study in the L and LF groups and among the treatment groups at the end of the study (Tables 13 and 14). As well, no differences in AUC_G, AUC_I, or AUC_{GI} were seen at any time point during the study (Tables 13 and 14). The one exception was AUC_G, which was lower in the L-12 group compared to the L-20, LF-12, and LF-20 groups (Table 13).

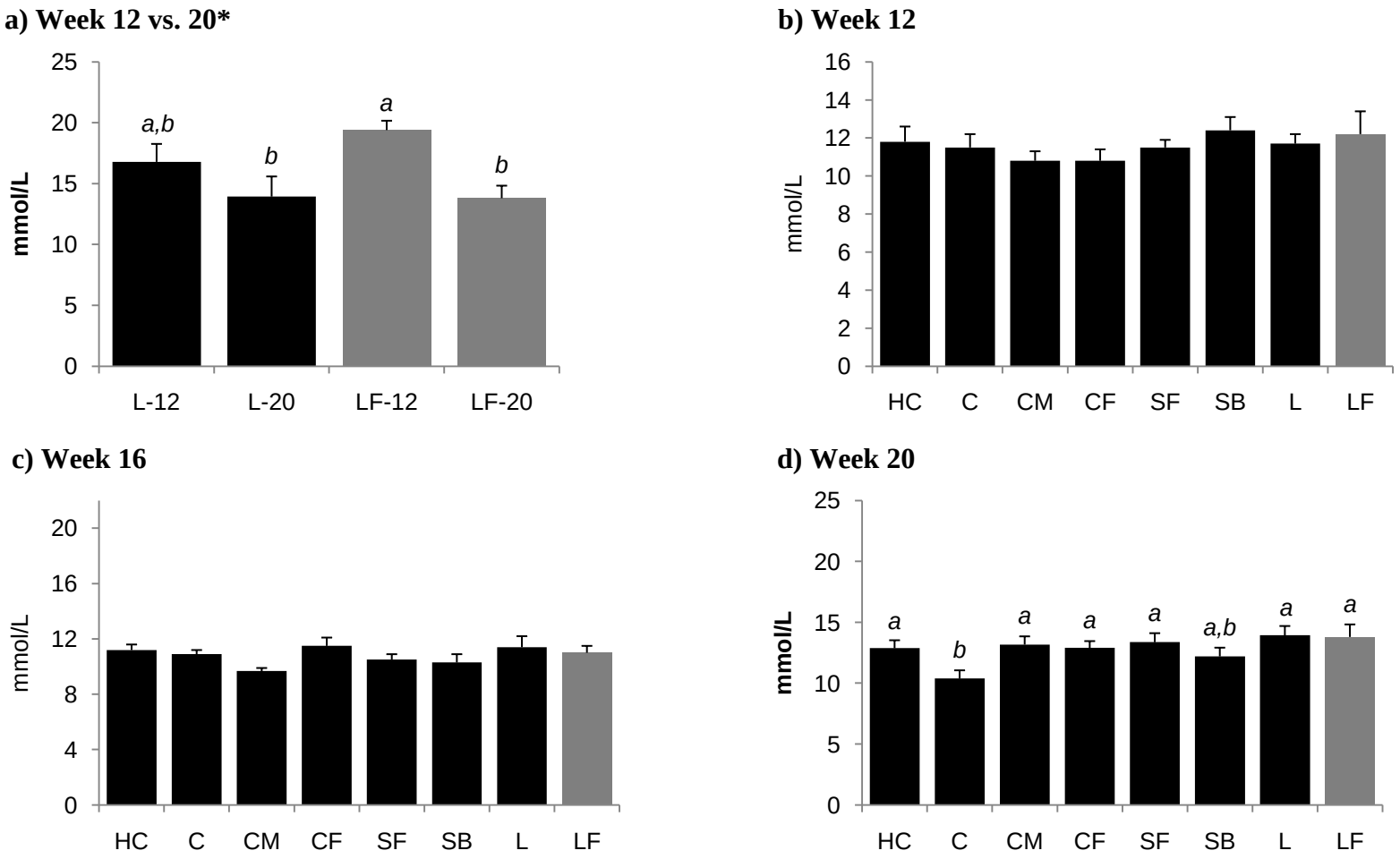


Figure 16. Serum glucose. Week 12 vs. 20 (a), Week 12 (b), Week 16 (c), Week 20 (d). Figures a) and d) represent serum collected from trunk blood after 12 hour fast; figures b) and c) represent serum collected from jugular blood after a 5 hour fast. Data expressed as mean ± SEM (n=10 rats per group). Different superscripts indicate significant differences ($p<0.05$) by Duncan's multiple range test. An asterisk (*) indicates data were log transformed prior to ANOVA. See Tables 7 and 9 for abbreviations.

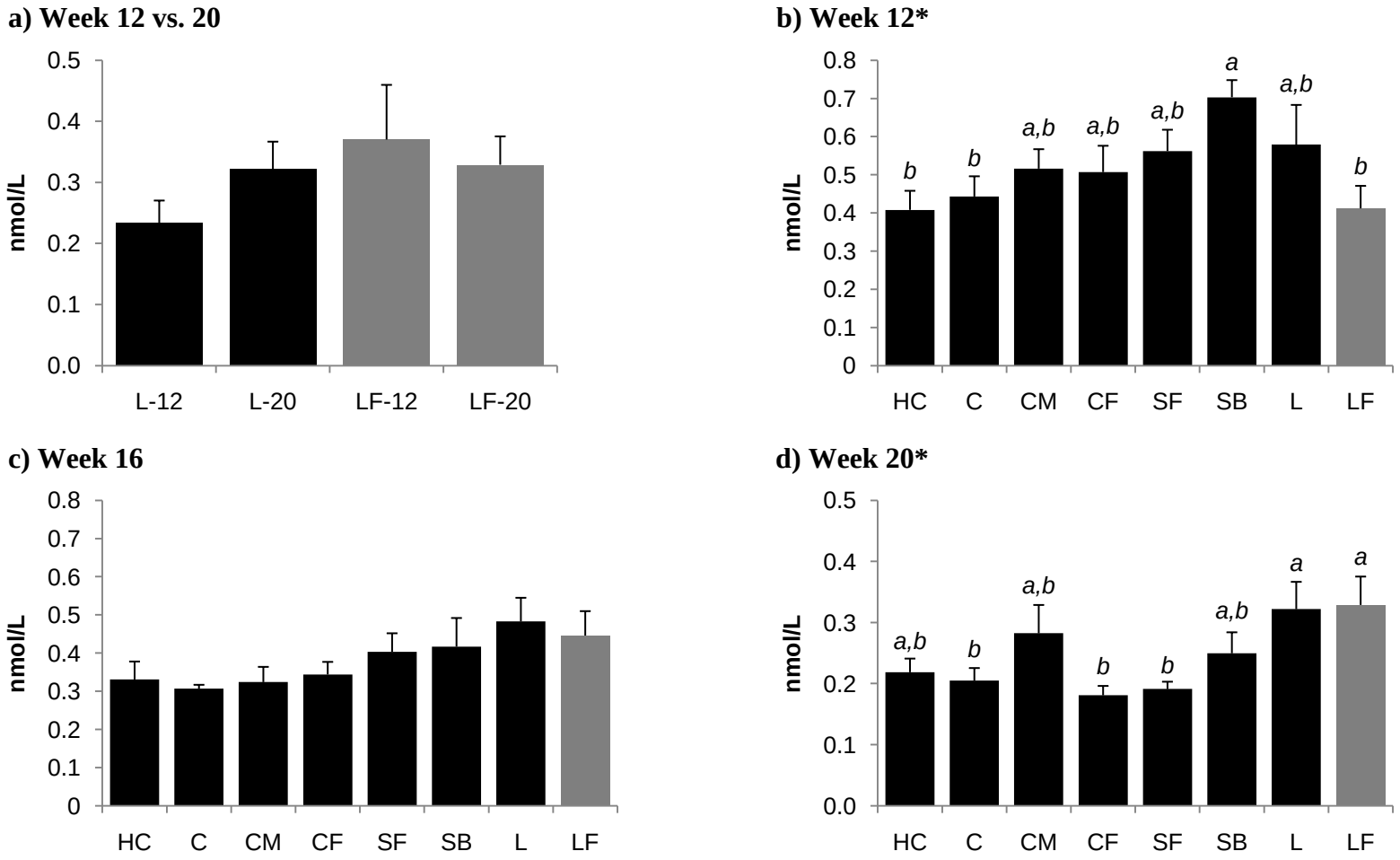


Figure 17. Serum insulin. Week 12 vs. 20 (a), Week 12 (b), Week 16 (c), Week 20 (d). Data expressed as mean ± SEM (n=8-10 rats per group). Figures a) and d) represent serum collected from trunk blood after a 12 hour fast; figures b) and c) represent serum collected from jugular blood after a 5 hour fast. Different superscripts indicate significant differences ($p<0.05$) by Duncan's multiple range test. An asterisk (*) indicates data were log transformed prior to ANOVA. See Tables 7 and 9 for abbreviations.

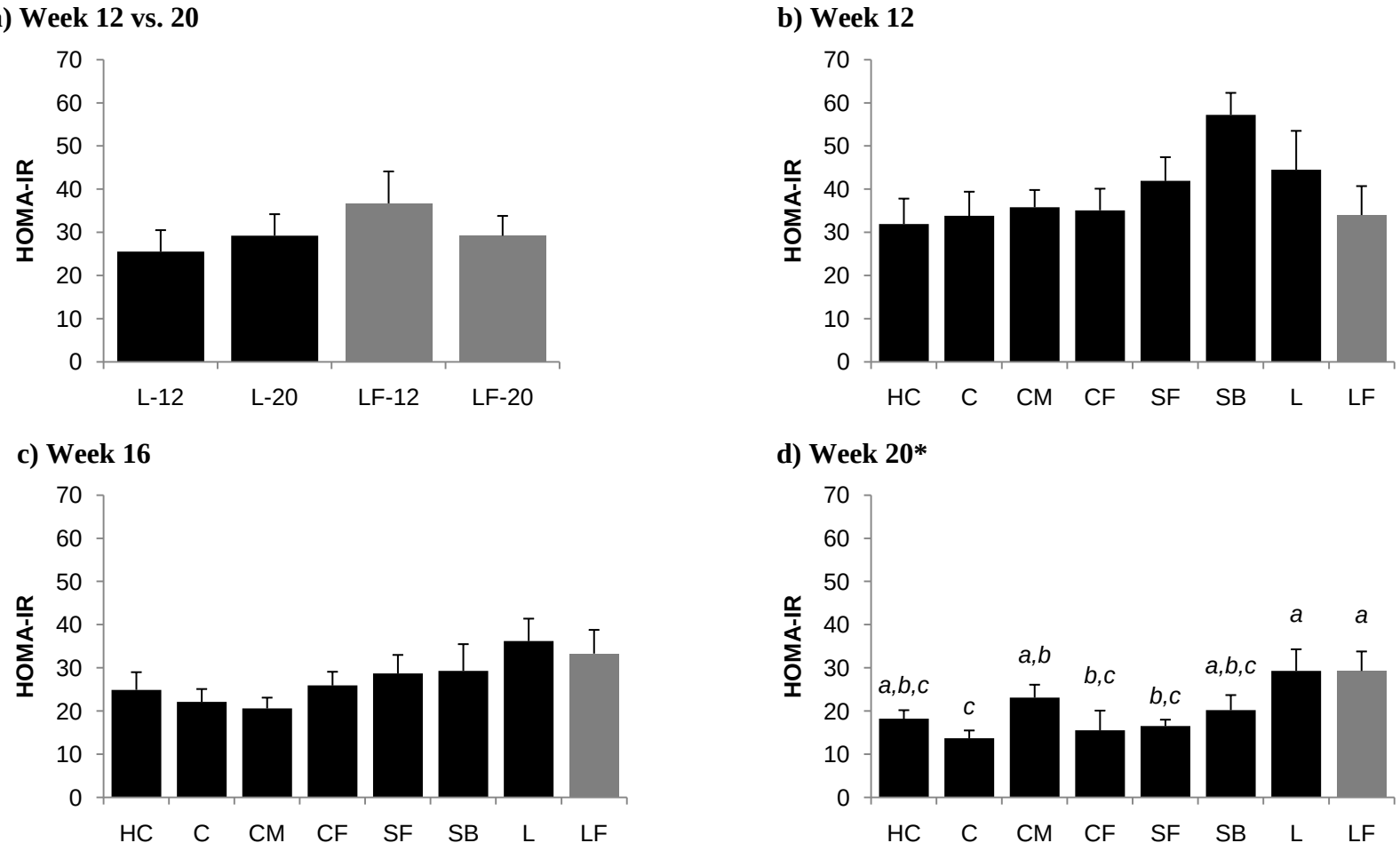


Figure 18. HOMA-IR. Week 12 vs. 20 (a), Week 12 (b), Week 16 (c), Week 20 (d). Data expressed as mean ± SEM (n=8-10 rats per group). Figures a) and d) represent serum collected from trunk blood after a 12 hour fast; figures b) and c) represent serum collected from jugular blood after a 5 hour fast. Different superscripts indicate significant differences ($p<0.05$) by Duncan's multiple range test. An asterisk (*) indicates data were log transformed prior to ANOVA. See Tables 7 and 9 for abbreviations.

Table 11. Insulin tolerance test glucose - week 10 vs. 18

	L-12	L-20	LF-12	LF-20
Glucose (mmol/L)				
t=0 min	7.16 ± 0.13	7.25 ± 0.23	7.82 ± 0.24	7.06 ± 0.34
t=15 min	6.70 ± 0.27	7.18 ± 0.19	7.47 ± 0.17	7.50 ± 0.32
t=30 min	6.17 ± 0.27	6.40 ± 0.28	6.63 ±0.34	5.94 ± 0.11
t=45 min	5.84 ± 0.37	6.12 ± 0.35	6.27 ± 0.46	5.32 ± 0.13
t=60 min	5.76 ± 0.42	6.02 ± 0.38	6.40 ± 0.49	5.25 ± 0.21

All data expressed as mean ± SEM (n=9 or 10 rats/group). An absence of superscripts indicates no significant differences at the same time point. See Table 9 for abbreviations; min, minutes.

Table 12. Insulin tolerance test glucose - week 18

	HC	C	CM	CF	SF	SB	L	LF
Glucose (mmol/L)								
t=0 min	6.95 ± 0.18	7.01 ± 0.20	7.08 ± 0.33	7.28 ± 0.22	6.90 ± 0.15	7.27 ± 0.10	7.25 ± 0.23	7.06 ± 0.34
t=15 min	6.95 ± 0.27	7.00 ± 0.24	7.16 ± 0.28	6.63 ± 0.33	6.87 ± 0.15	6.90 ± 0.22	7.18 ± 0.19	7.50 ± 0.32
t=30 min	6.04 ± 0.27	6.46 ± 0.35	6.67 ± 0.46	6.00 ± 0.22	5.94 ± 0.24	6.08 ± 0.22	6.40 ±0.28	5.94 ± 0.11
t=45 min	5.87 ± 0.31	6.07 ± 0.46	6.39 ± 0.58	5.68 ± 0.37	5.94 ± 0.28	5.94 ± 0 .42	6.12 ± 0.35	6.32 ± 0.13
t=60 min	5.92 ± 0.43	5.95 ± 0.50	5.87 ± 0.65	5.41 ± 0.63	5.74 ± 0.34	5.92 ± 0.59	6.02 ± 0.38	5.25 ± 0.21

All data expressed as mean ± SEM (n=9 or 10 rats/group). An absence of superscripts indicates no significant differences at the same time point. See Tables 7 and 11 for abbreviations.

Table 13. Oral glucose tolerance test glucose, insulin, and glucose insulin index - week 11 vs. 19

	L-12	L-20	LF-12	LF-20
Glucose (mmol/L)				
t=0 min	7.38 ± 0.25	8.71 ± 0.43	8.16 ± 0.19	8.22 ± 0.31
t=15 min	9.00 ± 0.33	10.3 ± 0.5	9.67 ± 0.34	10.4 ± 0.4
t=30 min	8.15 ± 0.44	10.6 ± 0.5	9.10 ± 0.28	9.49 ± 0.38
t=60 min	8.38 ± 0.35	9.72 ± 0.39	9.13 ± 0.34	8.80 ± 0.43
AUC_G[1]	499 ± 17[b]	592 ± 22[a]	549 ± 14[a]	560 ± 19[a]
Insulin (nmol/L)				
t=0 min	0.495 ± 0.046	0.671 ± 0.115	0.610 ± 0.077	0.755 ± 0.158
t=15 min	0.963 ± 0.099	1.01 ± 0.22	1.04 ± 0.11	1.13 ± 0.20
t=30 min	0.633 ± 0.047	0.893 ± 0.132	0.780 ± 0.083	0.925 ± 0.175
t=60 min	0.655 ± 0.041	0.785 ± 0.131	0.711 ± 0.061	0.972 ± 0.238
AUC_I	42.2 ± 2.2	52.1 ± 7.3	48.4 ± 4.4	58.0 ± 10.5
AUC_{GI}	21.3 ± 1.8	26.9 ± 3.0	29.7 ± 5.5	33.0 ± 6.6

All data expressed as mean ± SEM (n=9 or 10 rats/group). Different superscripts indicate significant differences ($p<0.05$) by Duncan's multiple range test. See table Tables 9 and 11 for abbreviations; AUC_G, area under the curve for glucose; ACU_I, area under the curve for insulin; AUC_{GI}, glucose insulin index.

[1]Data were log transformed prior to ANOVA.

Table 14. Oral glucose tolerance test glucose, insulin, and glucose insulin index - week 19

	HC	C	CM	CF	SF	SB	L	LF
Glucose (mmol/L)								
t=0 min	8.06 ± 0.14	7.87 ± 0.25	7.80 ± 0.28	8.04 ± 0.35	8.18 ± 0.28	7.85 ± 0.24	8.71 ± 0.43	8.22 ± 0.31
t=15 min	10.2 ± 0.4	10.1 ± 0.4	9.31 ± 0.33	9.55 ± 0.47	9.59 0.33	9.51 ± 0.34	10.3 ± 0.5	10.4 ± 0.4
t=30 min	9.42 ± 0.33	9.48 ± 0.37	8.54 ± 0.37	8.64 ± 0.40	8.78 ± 0.37	9.03 ± 0.24	10.6 ± 0.5	9.49 ± 0.38
t=60 min	8.85 ± 0.33	9.36 ± 0.33	8.90 ± 0.56	8.80 ± 0.95	8.91 ± 0.33	9.06 ± 0.23	9.72 ± 0.39	8.80 ± 0.43
AUC_G	558 ± 16	564 ± 16	524 ± 20	530 ± 21	536 ± 16	541 ± 13	592 ± 22	561 ± 19
Insulin (nmol/L)								
t=0 min	0.459 ± 0.087	0.477 ± 0.030	0.542 ± 0.053	0.588 ± 0.042	0.561 ± 0.049	0.464 ± 0.042	0.671 ± 0.115	0.755 ± 0.158
t=15 min	0.930 ± 0.167	0.889 ± 0.125	0.917 ± 0.106	1.01 ± 0.09	0.892 ± 0.106	0.719 ± 0.074	1.01 ± 0.22	1.13 ± 0.20
t=30 min	0.716 ± 0.148	0.558 ± 0.048	0.684 ± 0.091	0.757 ± 0.065	0.641 ± 0.042	0.634 ± 0.035	0.893 ± 0.132	0.925 ± 0.175
t=60 min	0.649 ± 0.170	0.606 ± 0.042	0.628 ± 0.093	0.551 ± 0.045	0.538 ± 0.044	0.558 ± 0.035	0.785 ± 0.131	0.972 ± 0.238
AUC_I	43.2 ± 8.7	38.6 ± 2.8	46.6 ± 3.9	44.8 ± 3.1	40.1 ± 2.8	36.9 ± 2.0	52.1 ± 7.3	58.0 ± 10.5
AUC_{GI}	24.8 ± 6.2	22.0 ± 2.0	22.6 ± 2.6	23.8 ± 2.0	21.8 ± 1.7	20.0 ± 1.4	31.1 ± 5.1	33.0 ± 6.6

All data expressed as mean ± SEM (n=9 or 10 rats/group). An absence of superscripts indicates no significant differences at the same time point. See Tables 7, 11 and 13 for abbreviations.

Lipidemia

a) Serum free fatty acids

Serum FFAs did not change over the course of the study, nor did they differ between the L and LF groups at the same time point (Figure 19). Similarly, at the end of the study, there were no differences in serum FFAs among all groups (Figure 20).

b) Serum triglycerides

Serum TGs increased over the course of the study but did not differ, at the same time point, between the L and LF groups (Figure 21 a).

Significant differences were not expected at week 12 as this was the beginning of the treatment phase. However, the C group had lower serum TGs than the L group (Figure 21 b). Additionally, the LF group had lower serum TGs than the L group (Figure 21 b) which does not agree with the L-12 and LF-12 results.

At week 16, all groups had lower serum TGs than the L group. Additionally, the SF and SB groups had lower serum TGs than the LF group (Figure 21 c).

At week 20, the L and LF groups had similar serum TGs. The C, CF, SF, and SB groups had lower serum TGs than the L group; out of these groups the SB group was lower than the C group (Figure 21 d). Values for both serum FFAs and TGs are tabulated in Appendix 5.

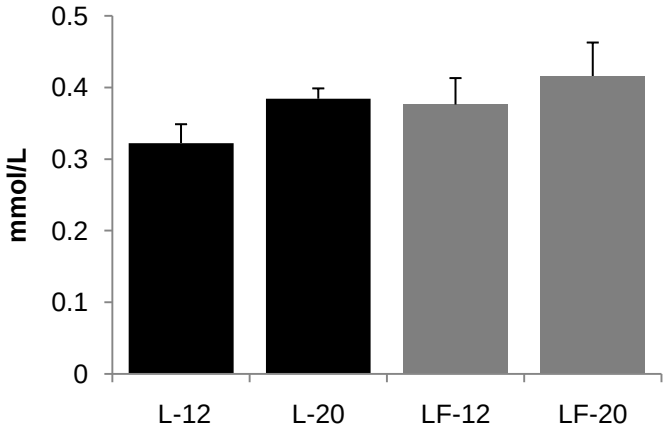


Figure 19. Serum free fatty acids - week 12 vs. 20. Data expressed as mean ± SEM (n=10 rats per group). An absence of superscripts indicates no significant differences. See Table 9 for abbreviations.

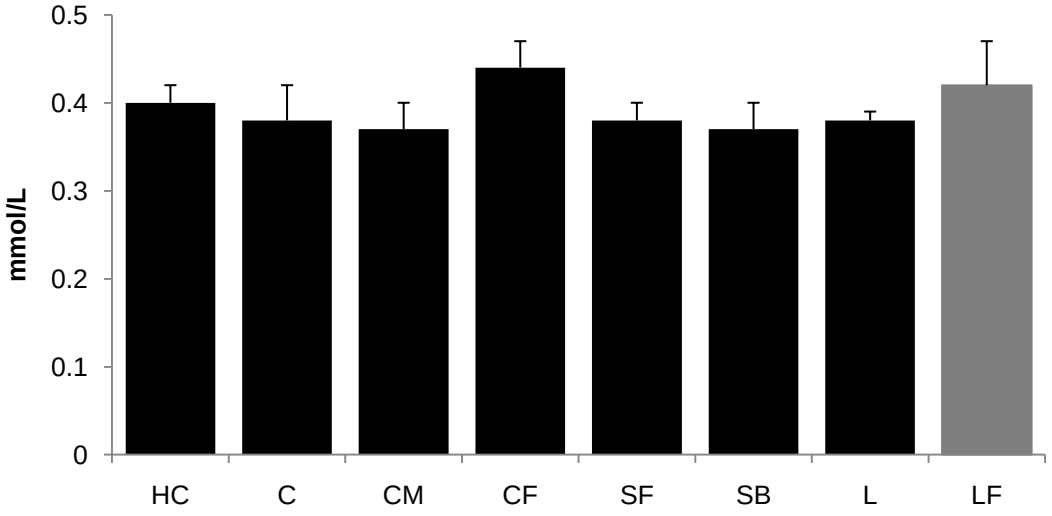


Figure 20. Serum free fatty acids - week 20. Data expressed as mean ± SEM (n=10 rats per group). An absence of superscripts indicates no significant differences. See Table 7 for abbreviations.

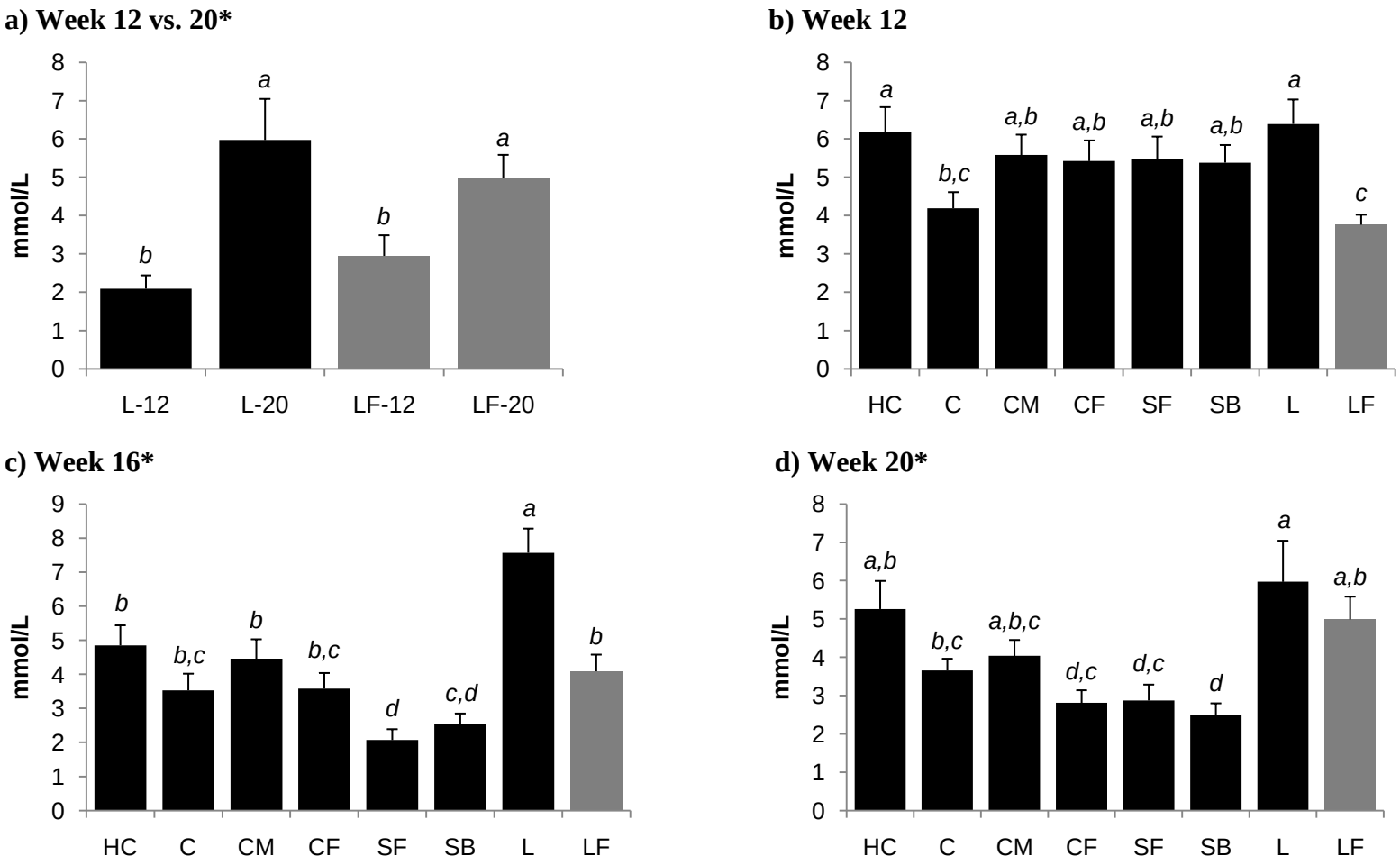


Figure 21. Serum triglycerides. Week 12 vs. 20 (a), Week 12 (b), Week 16 (c), Week 20 (d). Data expressed as mean ± SEM (n=9 or 10 rats per group). Figures a) and d) represent serum collected from trunk blood after a 12 hour fast; figures b) and c) represent serum collected from jugular blood after a 5 hour fast. Different superscripts indicate significant differences ($p<0.05$) by Duncan's multiple range test. An asterisk (*) indicates data were log transformed prior to ANOVA. See Tables 7 and 9 for abbreviations.

Muscle Phospholipid Fatty Acid Composition

The L-12 and LF-12 groups differed from each other in C16:0 (Figure 22 a), C18:1n7 (Figure 23 c), C18:2n6 (Figure 24 a), C18:3n3 (Figure 25 a), and C22:6n3 (Figure 25 e) composition where the L-12 group was lower and in C18:n9 (Figure 23 a) composition where the L-12 group was higher. The L and LF groups showed no changes in their gastrocnemius muscle PL-FA composition over the course of the study (Tables 15 and 17, Figures 22 a and c, 23 a and c, 24 a and c, and 25 a, c, and e). Therefore, the results focus on the treatment phase.

a) Major groupings

At the end of the study, SFAs were highest in the L group which did not differ from the SF, and SB groups; MUFAs were highest in the HC group which did not differ from the C, CM, CF, and SB groups; PUFAs were highest in the SF and LF groups which did not differ from the HC and CF groups; n-6 PUFAs were highest in the SF group; n-3 PUFAs were highest in the CF group which did not differ from the C group; n-9 MUFAs were highest in the HC and CM groups which did not differ from the C and CF groups (Table 16). For the most part, these results are what were expected based on the dietary fatty acid composition. Unexpected results include the high amounts of SFA in the gastrocnemius muscle PLs of the SF and SB groups and the high MUFAs in the SB group.

b) Fatty acid ratios

The FA ratios in the treatment groups also showed expected patterns; the n-6/n-3 PUFA ratio was lowest in the CF group, but did not differ from the C and CM groups and the SF group had the highest n-6/n-3 ratio. Additionally, the n-9 MUFA/n-6 PUFA ratio

was lowest in the SF group. Surprisingly, the PUFA/SFA ratio did not differ among groups (Table 18).

c) Fatty acids of interest

i)Saturated fatty acids

The SFAs of highest abundance in the gastrocnemius muscle PLs were C16:0 (palmitic acid) and C18:0 (stearic acid). At the end of the study, C16:0 was similar among all treatment groups, however, the SB and LF groups had higher C16:0 than the HC, C, and CF groups (Figure 22 b). C18:0 was lower in the HC, C, CM, CF, and LF groups compared to the L and SF groups (Figure 22 d).

ii) Monounsaturated fatty acids

MUFAs of interest in the gastrocnemius muscle PLs were C18:1n9 (oleic acid) and C18:1n7 (*trans* vaccenic acid). Compared to the L group, the SF and LF groups had lower, while the HC and CM groups had higher, amounts of C18:1n9 (Figure 23 b) at the end of the study. C18:1n7 was not different among any of the groups at week 20 (Figure 23 d).

iii) N-6 polyunsaturated fatty acids

C18:2n6 (LA) was higher in the CF, SF, SB and LF groups compared to the L group at week 20 (Figure 24 b) and the SF group had the highest LA compared to all groups except the LF group. C20:4n6 (AA), the long-chain counterpart to C18:2n6, was lower in all groups compared to the L group; exceptions to this were the HC group, which did not differ from the L group, and SF which was higher in C20:4n6 than the L group (Figure 24 d).

iv) N-3 polyunsaturated fatty acids

C18:3n3 (ALA), C20:5n3 (EPA), and C22:6n3 (DHA) were the n-3 PUFAs of interest in the gastrocnemius muscle PLs. As expected, the CF group had the highest ALA and EPA, however, only the SF group had lower C22:6n3 than the CF group (Figure 25 b, d, and f). ALA closely followed the n-3 pattern of the diets where CF > LF, SB, C ≥ CM ≥ HC, L ≥ SF. EPA followed a similar pattern except the SB and LF groups were lower than the CM group: CF > C > CM ≥ SB, L, LF ≥ HC > SF. DHA content did not follow this pattern; none of the canola-based groups differed from the CF group in DHA composition but the HC group had lower DHA than the C and CM groups.

All fatty acids values are tabulated in Appendix 6.

Table 15. Major fatty acid groupings in gastrocnemius muscle tissue phospholipids - week 12 vs. 20

	L-12	L-20	LF-12	LF-20
SFA	41.7 ± 1.7	41.8 ± 0.6	40.7 ± 0.7	39.5 ± 0.3
MUFA[1]	11.1 ± 1.5[a]	11.2 ± 1.2[a]	7.52 ± 0.13[b]	8.29 ± 0.44[a,b]
PUFA[2]	47.3 ± 1.8[b]	47.0 ± 1.7[b]	51.8 ± 0.6[a]	52.3 ± 0.7[a]
n-6	33.0 ± 1.2	32.6 ± 1.6	34.7 ± 0.4	36.2 ± 0.6
n-3	14.3 ± 0.8[b]	14.4 ± 0.4[b]	17.1 ± 0.4[a]	16.0 ± 0.2[a]
n-9[2]	6.41 ± 0.58[a]	7.10 ± 0.42[a]	4.13 ± 0.17[b]	4.15 ± 0.17[b]

All data expressed as mean ± SEM (n=5 rats/group) as g/100 g FA. Superscripts indicate significant differences ($p<0.05$) by Duncan's multiple range test. See Table 9 for abbreviations; SFA, saturated fatty acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid.

[1]Non-parametric testing was used for statistical analyses.

[2]Data were log transformed prior to ANOVA.

Table 16. Major fatty acid groupings in gastrocnemius muscle tissue phospholipids - week 20

	HC	C	CM	CF	SF	SB	L	LF
SFA	36.7 ± 0.6^{d}	38.3 ± 0.8^{c,d}	39.0 ± 0.5^{c}	38.1 ± 0.8^{c,d}	41.3 ± 0.9^{a,b}	41.1 ± 0.5^{a,b}	41.8 ± 0.6^{a}	39.5 ± 0.3^{b,c}
MUFA[1]	14.8 ± 0.7^{a}	13.9 ± 0.8^{a,b}	14.4 ± 0.7^{a,b}	12.9 ± 0.9^{a,b}	6.88 ± 0.23^{d}	11.5 ± 2.9^{a,b,c}	11.2 ± 1.2^{b,c}	8.29 ± 0.44^{c,d}
PUFA[1]	48.4 ± 0.8^{a,b}	47.8 ± 0.7^{b}	46.6 ± 0.5^{b}	49.0 ± 1.0^{a,b}	51.8 ± 0.8^{a}	47.4 ± 3.0^{b}	47.0 ± 1.7^{b}	52.5 ± 0.7^{a}
n-6	34.4 ± 0.8^{b,c}	31.2 ± 0.7^{c}	30.9 ± 0.5^{c}	31.5 ± 0.6^{c}	41.9 ± 1.2^{a}	32.4 ± 2.5^{b,c}	32.6 ± 1.6^{b,c}	36.2 ± 0.6^{b}
n-3[1]	14.0 ± 0.2^{e}	16.5 ± 0.2^{a,b}	15.7 ± 0.3^{b,c}	17.4 ± 0.4^{a}	9.88 ± 0.41^{f}	15.0 ± 0.6^{c,d}	14.4 ± 0.4^{d,e}	16.0 ± 0.2^{b,c}
n-9[1]	10.6 ± 0.1^{a}	8.54 ± 0.31^{a,b}	9.70 ± 0.83^{a}	9.08 ± 0.58^{a,b}	3.88 ± 0.19^{c}	6.83 ± 2.00^{b}	7.10 ± 0.42^{b}	4.14 ± 0.17^{c}

All data expressed as mean ± SEM (n=5 rats/group) as g/100 g FA. Superscripts indicate significant differences ($p<0.05$) by Duncan's multiple range test. See Table 7 and 15 for abbreviations.
[1]Non-parametric testing was used for statistical analyses.

Table 17. Fatty acid ratios in gastrocnemius muscle tissue phospholipids - week 12 vs. 20

	L-12	L-20	LF-12	LF-20
PUFA/SFA[1]	1.15 ± 0.08	1.28 ± 0.04	1.13 ± 0.06	1.32 ± 0.03
n-6/n-3	2.32 ± 0.10	2.27 ± 0.10	2.04 ± 0.04	2.27 ± 0.05
n-9/n-6[1]	0.196 ± 0.021[a]	0.222 ± 0.025[a]	0.119 ± 0.005[b]	0.115 ± 0.005[b]
C20:4n6/C20:3n6	13.1 ± 0.3[b]	13.0 ± 0.6[b]	14.9 ± 0.6[a]	15.5 ± 0.5[a]

All data expressed as mean ± SEM (n=5 rats/group) as g/100 g FA. Superscripts indicate significant differences ($p<0.05$) by Duncan's multiple range test. See Table 9 and 15 for abbreviations.
[1]Non-parametric testing was used for statistical analyses.

Table 18. Fatty acid ratios in gastrocnemius muscle tissue phospholipids - week 20

	HC	C	CM	CF	SF	SB	L	LF
PUFA/SFA[1]	1.32 ± 0.04	1.25 ± 0.04	1.20 ± 0.02	1.29 ± 0.05	1.26 ± 0.05	1.16 ± 0.08	1.13 ± 0.06	1.32 ± 0.03
n-6/n-3[2]	2.46 ± 0.07^{b}	1.89 ± 0.04^{e}	1.97 ± 0.05^{d,e}	1.81 ± 0.02^{e}	4.29 ± 0.31^{a}	2.14 ± 0.12^{c,d}	2.27 ± 0.10^{b,c}	2.27 ± 0.05^{b,c}
n-9/n-6[1]	0.308 ± 0.011^{a}	0.274 ± 0.012^{a}	0.314 ± 0.024^{a}	0.289 ± 0.022^{a}	0.093 ± 0.005^{c}	0.242 ± 0.102^{a}	0.222 ± 0.025^{a,b}	0.115 ± 0.005^{b,c}
C20:4n6/C20:3n6[1]	13.3 ± 0.2^{c,d}	11.9 ± 0.4^{d,e}	11.1 ± 0.8^{e}	8.87 ± 0.42^{f}	20.8 ± 1.1^{a}	14.3 ± 1.0^{b,c}	13.0 ± 0.5^{c,d,e}	15.5 ± 0.5^{b}

All data expressed as mean ± SEM (n=5 rats/group) as g/100 g FA. Superscripts indicate significant differences ($p<0.05$) by Duncan's multiple range test. See Table7 and 15 for abbreviations.

[1]Non-parametric testing was used for statistical analyses.

[2]Data were log transformed prior to ANOVA.

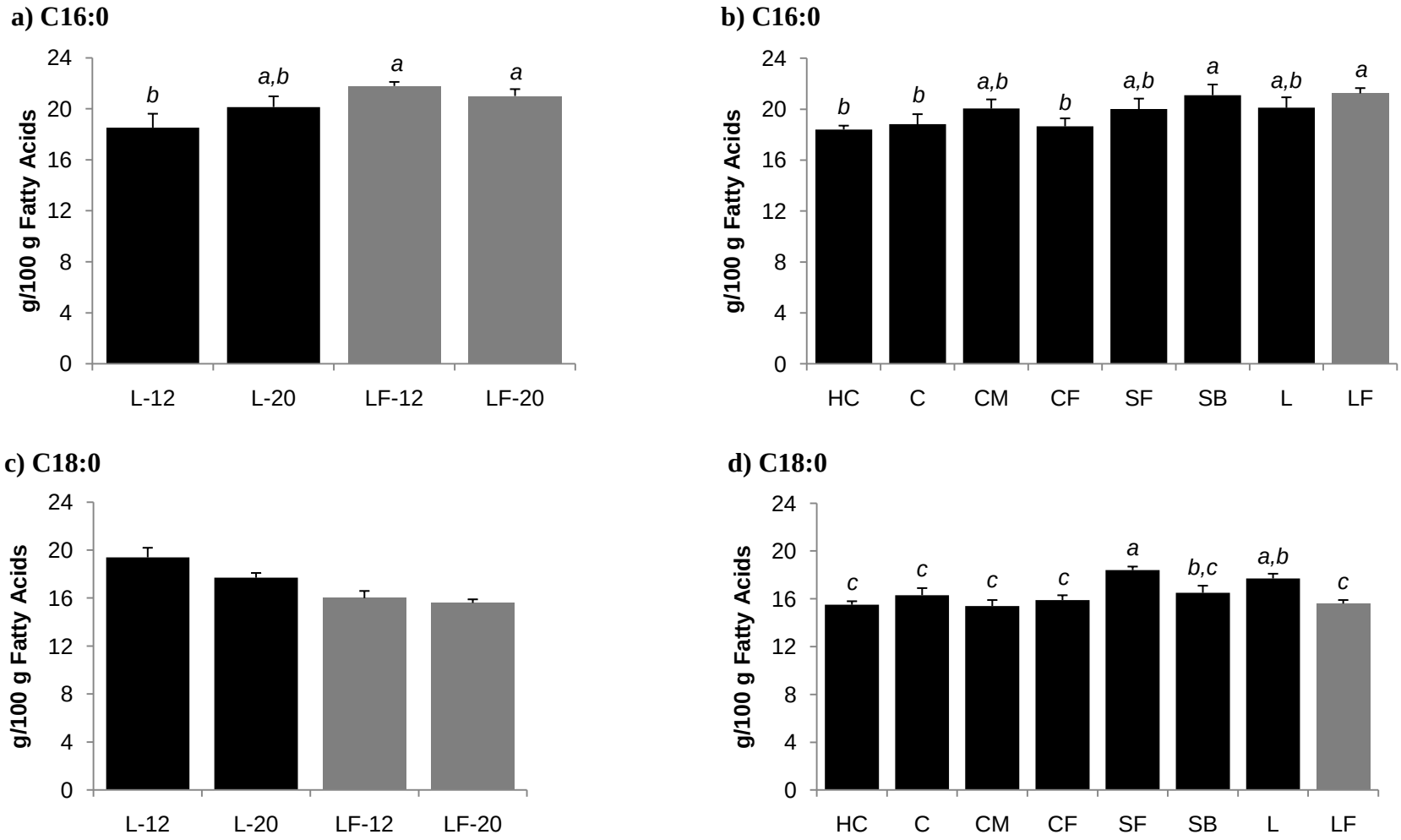


Figure 22. SFA composition of gastrocnemius muscle tissue phospholipids. C16:0 - Week 12 vs. 20 (a), C16:0 - Week 20 (b), C18:0 Week 12 vs. 20 (c), C18:0 - Week 20 (d). Data expressed as mean ± SEM (n=5 rats per group). Different superscripts indicate significant differences ($p<0.05$) by Duncan's multiple range test. See Tables 7 and 9 for abbreviations; g, grams.

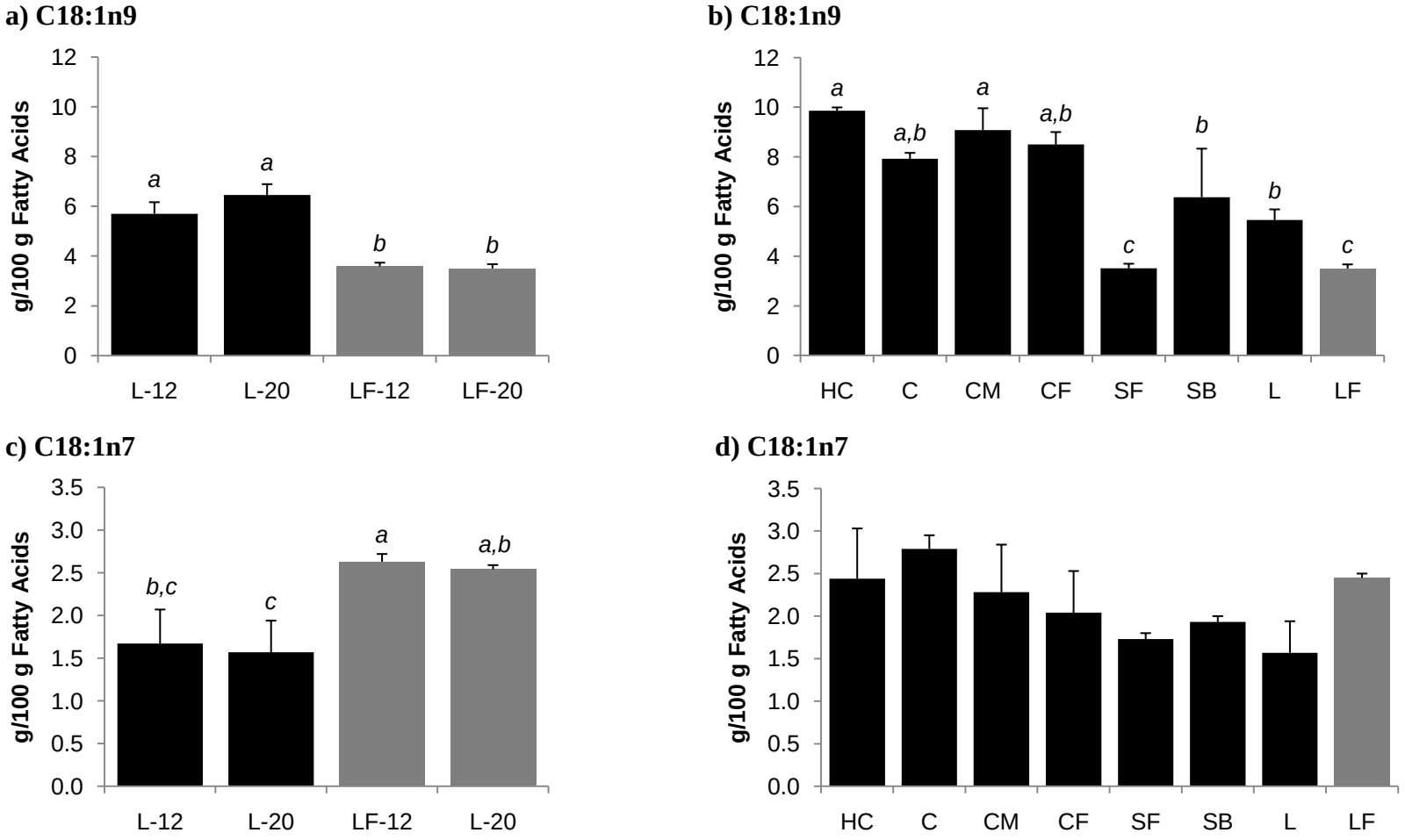


Figure 23. MUFA composition of gastrocnemius muscle tissue phospholipids. C18:1n9 - Week 12 vs. 20 (a), C18:1n9 - Week 20 (b), C18:1n7 Week 12 vs. 20 (c), C18:1n7 - Week 20 (d). Data expressed as mean ± SEM (n=5 rats per group). Different superscripts indicate significant differences ($p<0.05$) by Duncan's multiple range test. See Tables 7, 9 and 22 for abbreviations.

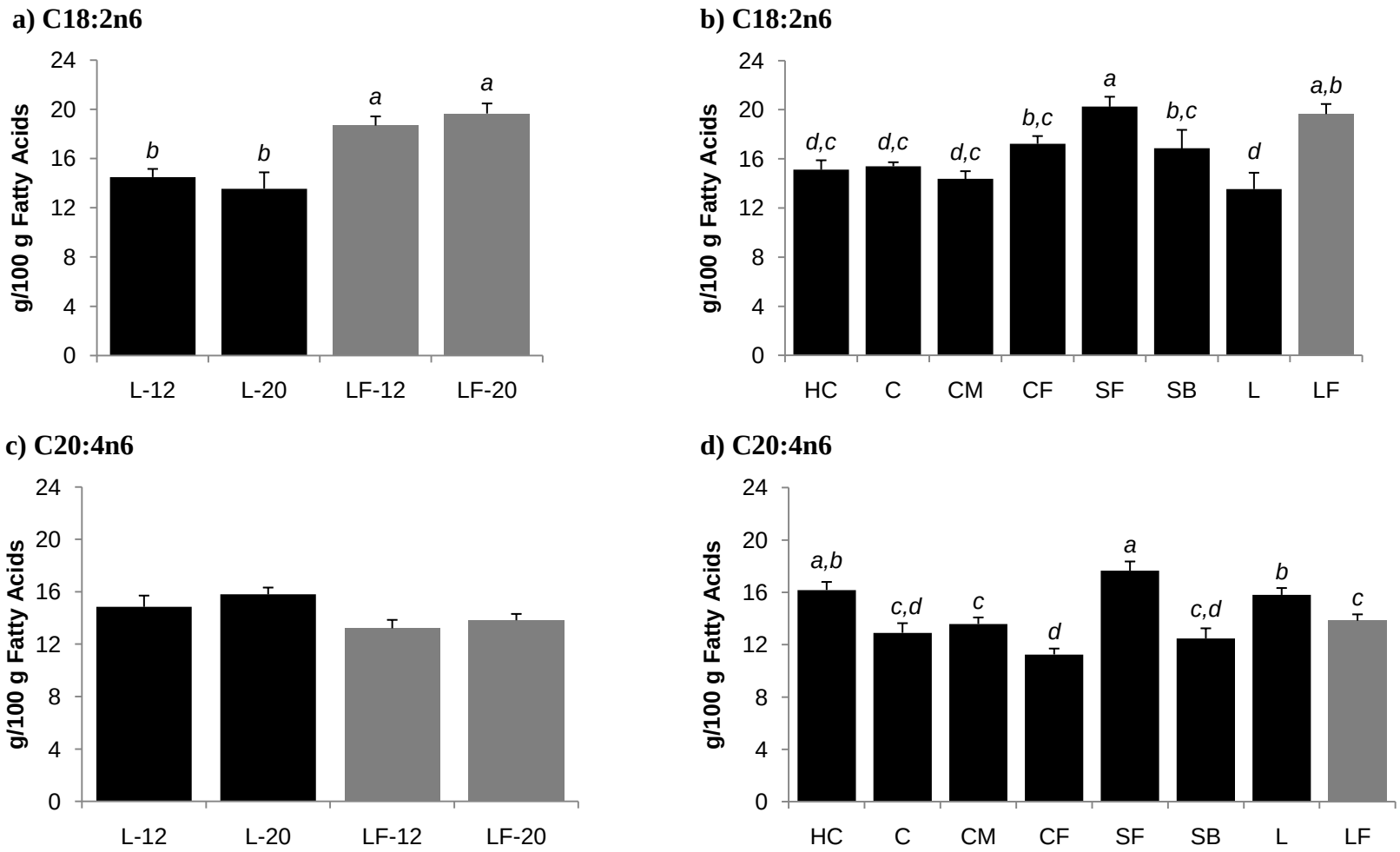
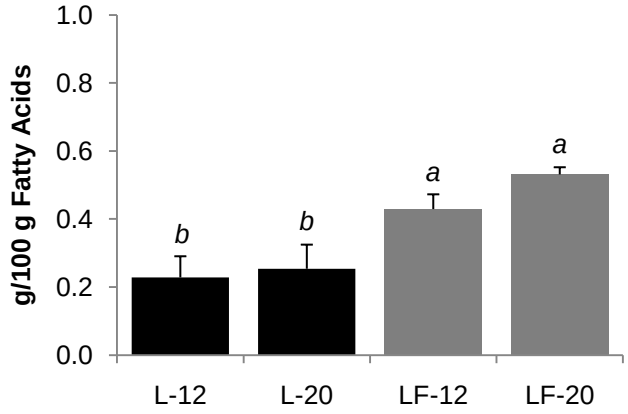
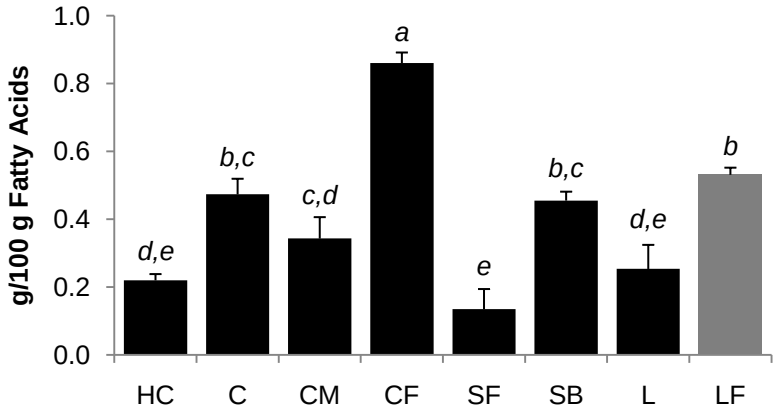


Figure 24. N-6 PUFA composition of gastrocnemius muscle tissue phospholipids. C18:2n6 - Week 12 vs. 20 (a), C18:2n6 - Week 20 (b), C20:4n6 Week 12 vs. 20 (c), C20:4n6 - Week 20 (d). Data expressed as mean ± SEM (n=5 rats per group). Different superscripts indicate significant differences ($p<0.05$) by Duncan's multiple range test. See Tables 7, 9, 22 for abbreviations.

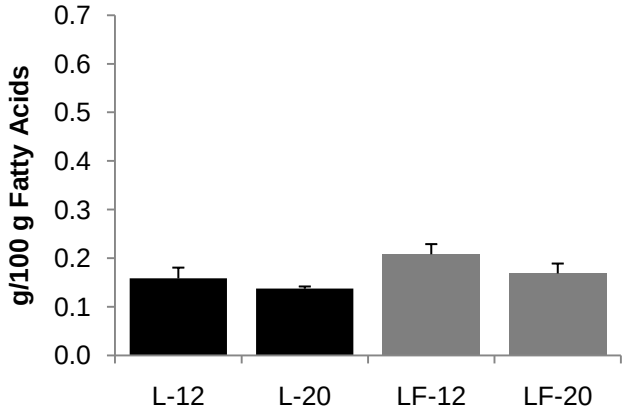
a) C18:3n3



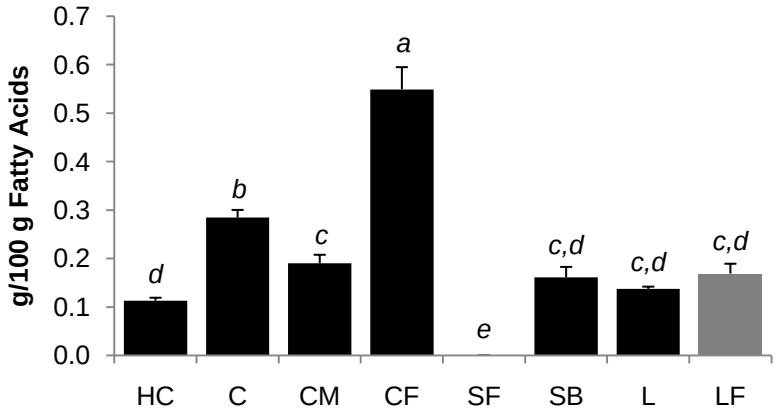
b) C18:3n3



c) C20:5n3



d) C20:5n3



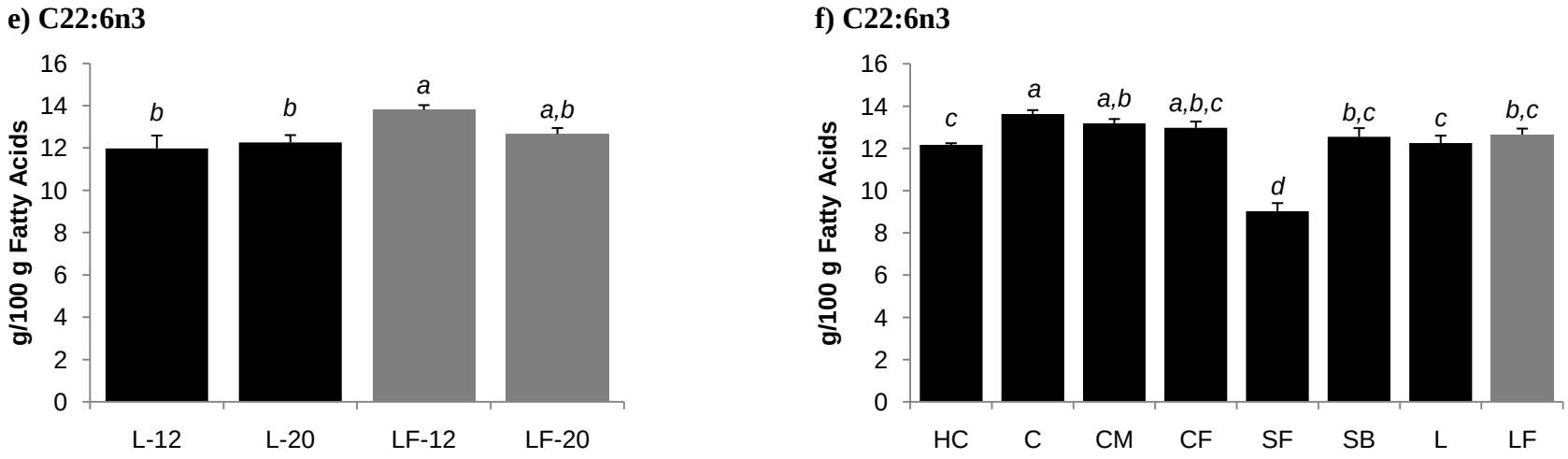


Figure 25. N-3 PUFA composition of gastrocnemius muscle tissue phospholipids. C18:3n3 - Week 12 vs. 20 (a), C18:3n3 - Week 20 (b), C20:5n3Week 12 vs. 20 (c), C20:5n3 - Week 20 (d), C22:6n3 - Week 12 vs. 20 (e), C22:6n3 - Week 20 (f). Data expressed as mean ± SEM (n=5 rats per group). Different superscripts indicate significant differences ($p<0.05$) by Duncan's multiple range test. See Tables 7, 9, and 22 for abbreviations.

Molecular Markers of Insulin Signalling in Muscle

No differences were seen in insulin signalling protein phosphorylation or protein levels for pAkt Thr^{308}, pAkt Ser^{473}, and pSAPK/JNK (Thr^{183}/Tyr^{185}) (Figures 26 and 27).

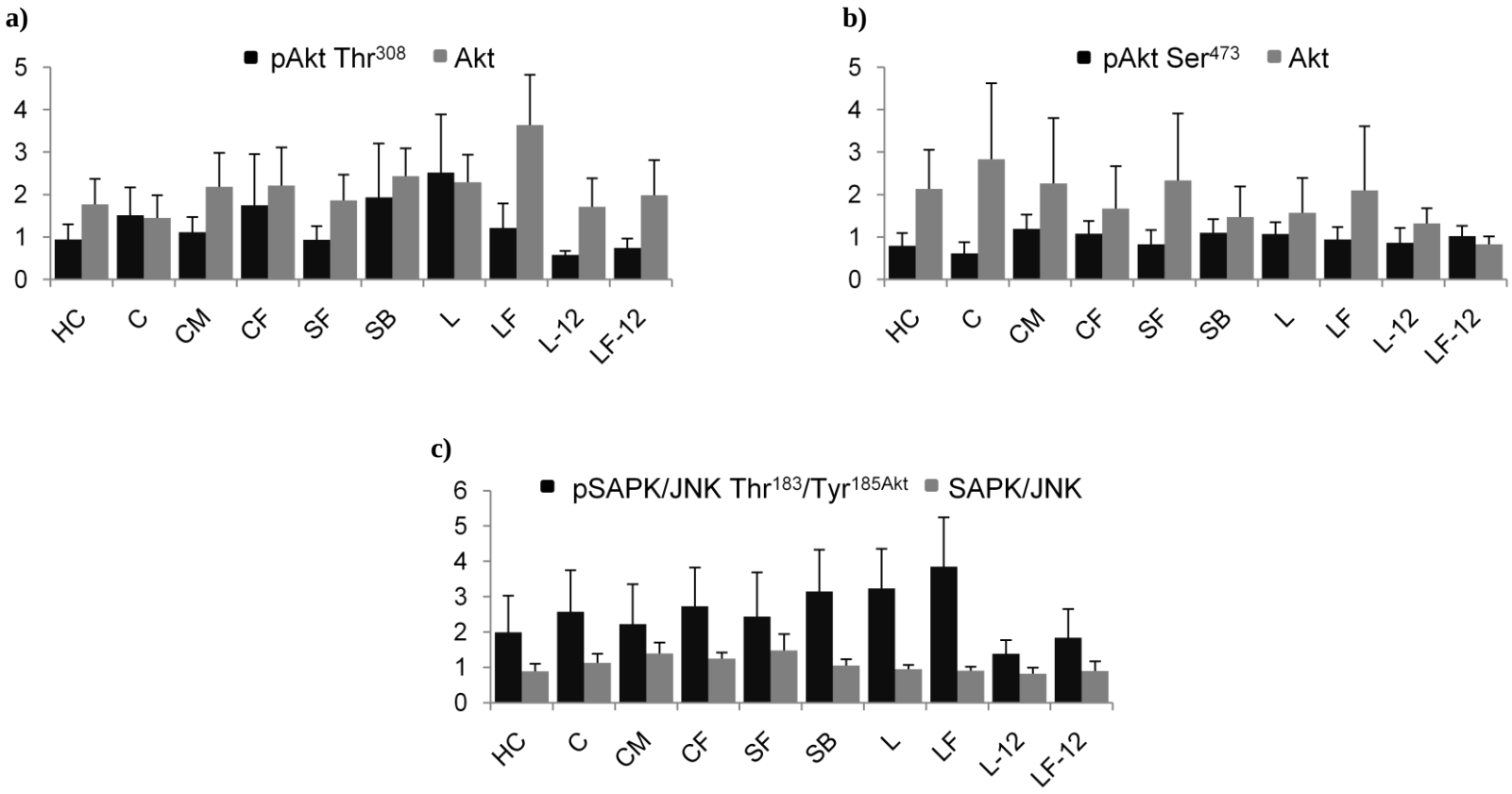


Figure 26. Relative insulin signalling protein phosphorylation and levels in gastrocnemius muscle. pAkt Thr^{308} (a), pAkt Ser^{473} (b), pSAPK/JNK Thr^{183}/Tyr^{185}. All data expressed as mean ± SEM (n=4 rats/group) in arbitrary units. All data were adjusted to control for differences in band intensities on different membranes. Phosphorylation and protein levels were adjusted to control for differences in protein loading. An absence of superscripts indicates no significant differences. There were no significant differences when week 20 and week 12 vs. 20 statistics were run separately. See Tables 7 and 9 for abbreviations. Thr^{308}, phosphorylated at threonine-308; Ser^{473}, phosphorylated at serine-473; Thr^{183}, phosphorylated at threonine-185; Tyr^{185}, phosphorylated tyrosine-185.

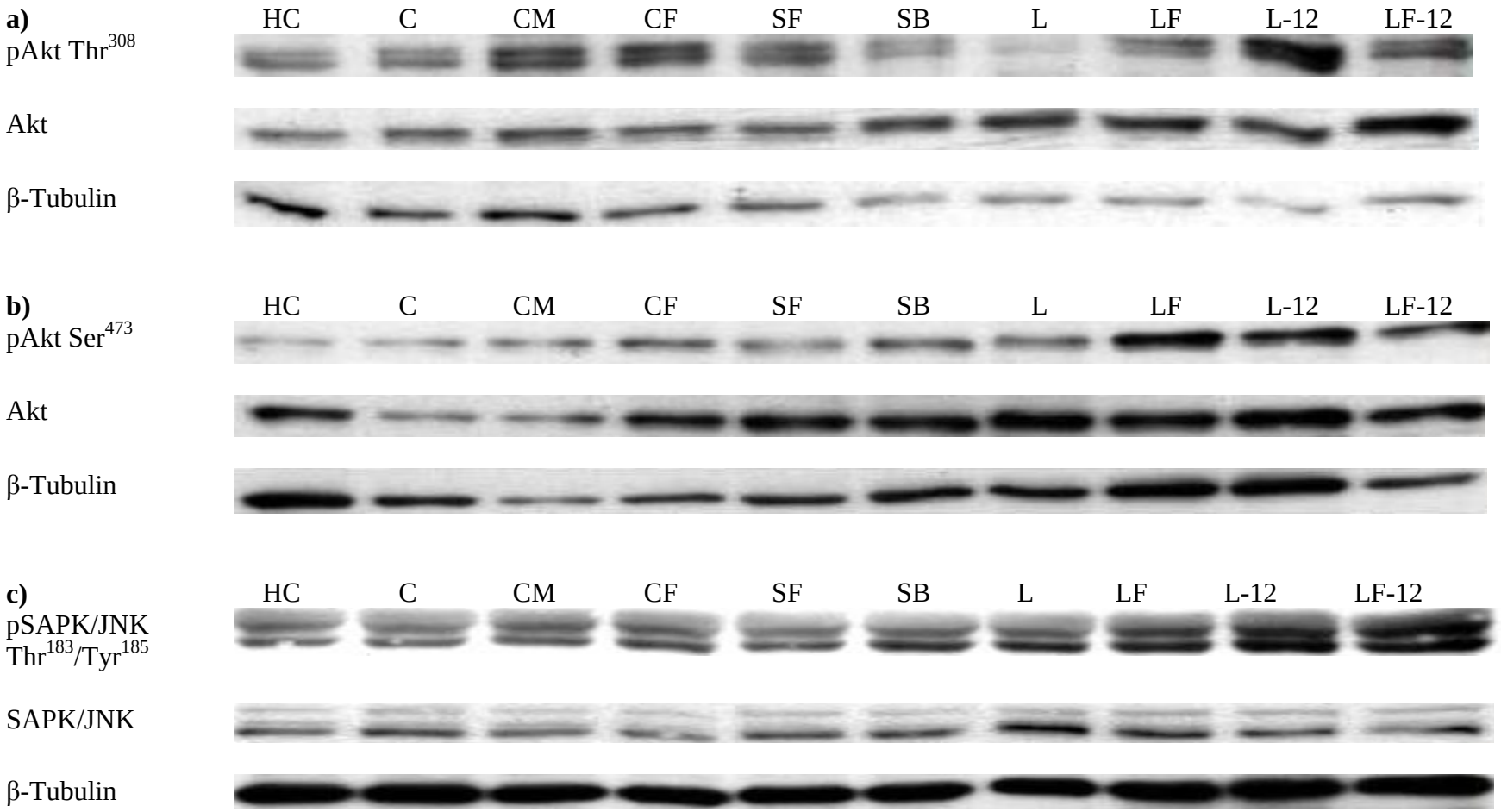


Figure 27. Representative blots of insulin signalling proteins. a) p-Akt Thr^{308}, b) p-Akt Ser^{473}, c) pSAPK/JNK Thr^{183}/Tyr^{185}. Figures obtained from Western Blot analyses. See Tables 7, 9 and 19 for abbreviations.

Correlations

a) Body weight/length vs. adipose tissue

Body weight/length measurements were compared to total visceral fat and individual fat pad masses (expressed as g/100 g bwt) (Table 19). The data from the L and LF groups terminated at weeks 12 and 20 were compared separately from the data from all groups terminated at week 20. All bwt/length vs. adipose tissue comparisons showed significant ($p<0.05$) positive associations except for when bwt/length was compared to epididymal fat in all groups from week 20. Additionally, strong positive associations ($r^2>0.6$) were seen when bwt/length was compared to total visceral fat, peri-renal fat, and mesenteric fat in the L and LF groups from weeks 12 and 20. However, only bwt/length vs. mesenteric fat, in all groups at week 20, had a strong association ($r^2>0.6$).

b) Diet and phospholipid fatty acid composition vs. serum parameters

Diet FA composition in major groupings (SFA, MUFA, PUFA, n-6 PUFA, n-3 PUFA, and n-6/n-3 PUFA ratio) was compared with serum TGs, glucose, insulin, and HOMA-IR scores in all groups terminated at week 20. A similar comparison, in the L and LF groups from weeks 12 and 20 was not conducted as only two levels of fatty acid composition were seen and this appeared to override any correlation effect (data not shown). PL-FA composition was also investigated in this way, except data from the L and LF groups terminated at weeks 12 and 20 was included. Significant ($p<0.05$) correlations are shown in Table 20.

For the groups terminated at week 20, diet SFA were positively associated with serum TGs, insulin, and HOMA-IR while diet MUFAs were negatively associated with

serum insulin. Diet total PUFAs, n-6 PUFAs, and n-3 PUFAs were all negatively associated with serum TGs.

The PL n-3 PUFAs were positively associated with serum glucose in the L and LF rats terminated at weeks 12 and 20 while the n-6/n-3 PUFA ratio was negatively associated with serum glucose. Additionally, this n-6/n-3 PUFA and serum glucose negative association was strong (r^2>0.6).

For all groups terminated at week 20, PL MUFAs were negatively associated, while PL total PUFAs and n-6 PUFAs were positively associated, with serum insulin.

All correlation results are shown in Appendix 8.

Table 19. Correlations - body weight/length vs. adipose tissue

Time	Parameter 1	Parameter 2	r^2	p-value
Weeks 12 vs. 20[1]	bwt/length	total visceral fat/100 g bwt	0.830	<0.0001
		epididymal fat/100 g bwt	0.497	0.001
		peri-renal fat/100 g bwt	0.796	<0.0001
		mesenteric fat/100g bwt	0.822	<0.0001
Week 20[1]	bwt/length	total visceral fat/100 g bwt	0.553	<0.0001
		epididymal fat/100 g bwt	0.209	0.063
		peri-renal fat/100 g bwt	0.534	<0.0001
		mesenteric fat/100g bwt	0.619	<0.0001

All data analyzed using Pearson's Correlation. Values are considered significant when $p<0.05$.
[1]n=78 to 80, lard and low-fat groups terminated at weeks 12 and 20.
[2]n=40, all groups terminated at week 20.

Table 20. Correlations - diet and phospholipid fatty acid composition vs. serum parameters

Time	Parameter 1	Parameter 2	r^2	p-value
Week 20[1]	diet SFA	serum TGs	0.325	0.003
		serum insulin	0.292	0.011
		HOMA-IR	0.354	0.001
	diet MUFA	serum insulin	-0.269	0.019
	diet PUFA	serum TGs	-0.325	0.003
	diet n-6 PUFA	serum TGs	-0.255	0.024
	diet n-3 PUFA	serum TGs	-0.253	0.025
Week 12 vs. 20[2]	PL n-3 PUFA	serum glucose	0.555	0.011
	PL n6/n3 PUFA ratio	serum glucose	-0.603	0.005
Week 20[3]	PL MUFA	serum insulin	-0.393	0.018
	PL PUFA	serum insulin	0.369	0.027
	PL n-6 PUFA	serum insulin	0.343	0.041

All data analyzed using Pearson's Correlation. Significant values ($p<0.05$) are listed; Appendix 8 shows all correlation statistics conducted.
[1]n=75 to 80, all groups terminated at week 20.
[2]n=20, lard and low-fat groups terminated at weeks 12 and 20.
[3]n=36, all groups terminated at week 20.

XIV. DISCUSSION

Summary of Main Findings

The main findings of this study were that dietary oils (HC, C, CM, CF, SF, and SB) do not alter body weight or visceral adipose mass. At the end of the study, the C group had the lowest fasting serum glucose; the C, CF, and SF groups had the lowest fasting serum insulin; and the C group had the lowest HOMA-IR score. However, other measures of insulin resistance (ITTs and OGTTs) were not different among groups, nor did they worsen over the course of the study. Fasting serum FFAs did not differ among groups but fasting serum TGs were lowest in the SF group at the end of the study. Molecular markers of insulin signalling were also not different among groups. PL-FA composition of gastrocnemius muscle tissue followed a similar pattern to dietary FA composition; however, major FA groupings were more normalized in the muscle PLs compared to the diets. The robustness of the muscle PL PUFA/SFA ratios may explain the lack of differences in insulin resistance. These findings will be discussed in more details in the following sections.

Dietary Fatty Acid Composition

Overall, the dietary fatty acid compositions were similar to those listed in the literature (Canola Council of Canada, 2009a); aside from the L diet, all diets had SFA, OA, LA, and ALA contents within 5 g/100 g of the referenced values (Table 2 and Table 7) . These minor differences can likely be attributed to inherent variation between the referenced oils and the study oils. In fact, the technical data sheet for the high-linoleic

safflower oil showed that specific FA content can vary as much as 8 g/100 g between lots.

As for the L diet, the SFA content was 9 g/100 g higher and the OA content was 8 g/100 g lower, than expected. Although this may not seem like a large difference, it was a noticeable difference compared to the other diets. Recall that the L diet was made up of an approximate ratio of 10:1 lard:soybean oil; therefore, if the FA composition can vary by as much as 8 g/100 g, then, perhaps, the lard and soybean oil were both at the upper end in terms of SFA content and at the lower end in terms of OA content. As mentioned, only the diets were evaluated for their FA composition, so the exact FA composition of the lard itself is unknown. The soybean oil composition, as based on the SB diet, shows slightly higher SFA (by 1 g/100 g) and slightly lower MUFA (by 3 g/100 g) composition than expected. If the raw lard composition followed a similar or more exaggerated pattern, it would explain the observed differences.

Another point to note, which differentiates the L diet from the oil diets, is that the L diet did not mix as homogenously as the oil diets because of the solid consistency of lard. Because of this, the portion of the diet sample analyzed may have had a disproportionate amount of lard in it. This could have also skewed the FA composition result from what was expected, especially if the raw lard SFA content was at the high end and the MUFA content was at the low end of the expected range.

In general, the diets did represent the variety of FA composition that was desired for this study.

Obesity

Overall, it can be concluded that the degree of obesity increased over the course of the study as represented by comparing the L and LF groups terminated at week 12 and week 20. This is reflected in significant increases in body weight (Table 9), body weight/body length ratios (Figure 4), visceral fat mass (expressed as g/100 g body weight) (Figure 6), as well as decreases in gastrocnemius muscle mass (expressed as g/100 g body weight) (Figure 14). As mentioned previously, bwt/length is not validated as a measure of obesity in rats; however, correlation statistics from the current study do give this measure some degree of validity. Body weight/length ratios were positively associated with total visceral fat and individual fat pad masses (expressed as g/100 g bwt) except for the epididymal fat pad masses from rats terminated at week 20 (Table 19).

All high-fat treatment groups had similar body weights (Table 9), body weight/body length ratios (Figure 5), visceral fat mass (expressed as g/100 g body weight) (Figure 7), and gastrocnemius muscle mass (expressed as g/100 g body weight) (Figure 15) at the end of the study. This is consistent with the literature where plant-based sources of n-3 PUFAs were incorporated into the diets of rodents (Ikemoto *et al*., 1996; Okuno *et al*., 1997; Storlein *et al*., 1991). One exception is a study where rats fed a perilla oil-based diet had lower epididymal fat pad mass compared to rats fed a lard-based diet (Okuno *et al*., 1997), however, the ALA content of this diet was extremely high (61 g/100 g fatty acid) compared to the CF diet in the current study (20 g/100 g fatty acids) and a combined visceral fat mass was not given. On the whole, although the hypothesis for this study was that diets high in ALA would be beneficial to obesity and insulin resistance, it was expected that the benefits would be reflected in serum and molecular

parameters and not necessarily in body composition. Additionally, feed intake and feed efficiency ratio were similar among these groups which may explain the lack of differences.

Surprisingly, the L group had lower epididymal fat pad mass (expressed as g/100 g body weight) than two of the other high-fat groups (C and SF). Although the reason for this is unclear, it is also, likely, irrelevant since it did not have an effect on combined visceral fat mass (expressed as g/100 g body weight). However, it should be noted that epididymal fat may be the most responsive fat pad to dietary fat manipulation. Epididymal fat was the only fat pad that showed significant difference among high-fat fed groups at week 20 in the current study; this is in agreement with the study by Okuno *et al.* (1997) where epididymal, but not peri-renal, fat responded to different dietary oil interventions. Additionally, the epididymal fat pad was the only fat pad that did not have a significant association ($p<0.05$) with bwt/length ratios in rats terminated at week 20; when using data from the L and LF rats terminated at weeks 12 and 20, epididymal fat was associated with bwt/length ratios but the correlation was weak (r^2'0.6) compared to the other fat pads.

As expected, the LF group had the overall lowest visceral, epididymal, and peri-renal fat mass (all expressed as g/100 g body weight) (Figures 7, 9, and 11). Mesenteric fat pad mass, however, was not different among any of the groups which suggests it may be more resistant to dietary manipulation than the other fat pads (Figure 13).

It was unexpected that the LF group did not have significantly lower body weight or body length/weight ratios than the high-fat groups. This is potentially due to the macronutrient composition of the diets. The LF diet contained 25% of energy from fat

while other DIO studies employing a low-fat control diet, used 12-13% of energy from fat (Levin & Keesey, 1998; Madsen *et al*., 2010; Storlein *et al*., 1991) and the AIN-G diet contains 17% of energy from fat (Reeves, Nielsen, & Fahey, 1993). Furthermore, in two of these studies (Levin & Keesey, 1998; Madsen *et al.*, 2010) the high-fat diet compositions were closer to the current study's low-fat composition at 31-32% of energy from fat. Therefore, a low-fat diet with a lower percentage of energy from fat would have likely been a more representative control diet for this study. The reasons for not using a diet with a lower percentage of energy from fat were, first, to avoid having a high carbohydrate diet which might have detrimental effects to obesity and insulin resistance. Evidence has shown that when comparing a low-fat (or high-carbohydrate) diet to very low carbohydrate diet, the low-fat diet resulted in less insulin sensitivity (as measured by HOMA-IR in overweight and/or obese women) (Volek *et al*., 2004). Secondly, a fat composition that fits in with the acceptable macronutrient distribution range for humans, 20-35% of energy from fat (Trumbo *et al*., 2002), was desired for the present study.

Another interesting finding was that, although body weights were not different from weeks 12 to 20 (Table 10), weight gain was different among groups (Figure 3) during the treatment phase. In particular, the LF group had the greatest weight gain from week 12 to 20. It is unclear why this occurred but perhaps this model has a genetically predetermined weight range and therefore all groups gained the appropriate amount of weight to meet these "set" values. This is, however, speculation and should be investigated further. Additionally, the large weight gain in the LF group may explain why the LF group was not the healthy control it was expected to be; it was similar to the L group in a number of parameters including serum glucose, insulin, and HOMA-IR scores.

Insulin Resistance

Fasting serum glucose and insulin were used to calculate HOMA-IR scores which are more representative of insulin resistance than either of the serum values alone. This is because HOMA-IR combines both values to express a relationship between serum glucose and insulin which is, ultimately, the accepted criterion for determining insulin resistance (Matthews *et al*., 1985). Over the course of the study, the HOMA-IR scores did not increase in the L and LF groups (Figure 18 a) suggesting that insulin resistance, being a progressive condition, was not well established in this model by week 12 of the study. Additionally, it appears as though HOMA-IR scores in the treatment groups declined from week 12 to 16 to 20 (Figure 18 b, c, and d) which would indicate some sort of adaptation to the dietary interventions. It should be noted, though, that comparisons between treatment groups at weeks 12, 16, and 20 should be made with caution as blood collection methods were different at these times (jugular blood collected at week 12 and 16 after a 5 hour fast; trunk blood collected at week 20 after a 12 hour fast).

A comparison in serum glucose from different blood collection methods was performed on 10 “alternate” rats and showed serum glucose was lowest during saphenous blood collection, intermediate during jugular blood collection, and highest during trunk blood collection (see Appendix 7). To collect blood for these comparisons, saphenous blood was collected immediately before jugular blood collection (after a 5 hour fast) and trunk blood was collected the following morning (after a 12 hour fast). There are several potential explanations for differences in serum glucose concentrations. First, during jugular blood collection, rats were anaesthetized with isoflurane which has been shown to induce hyperglycemia (Zuurbier *et al*., 2008). Second, in the fasted state, hepatic

gluconeogenesis occurs; insulin inhibits gluconeogenesis while stimulating glucose uptake by tissues (Mahan & Escott-Stump, 2004). In a state of insulin resistance, during fasting, it is expected that serum glucose levels will be elevated due to reduced inhibition of hepatic gluconeogensis and reduced glucose uptake by tissues. The serum glucose concentrations from trunk blood collection in the current study agrees with this and suggests that, with an increased length of fasting, serum glucose levels become increasingly elevated especially in the presence of insulin resistance.

Looking at the week 20 data, if the L diet is to be considered the "unhealthy" control then the C, CF, and SF groups had healthier (lower) HOMA-IR scores at the end of the study (no differences were observed among groups at week 12 or 16) (Figure 18 b, c, and d). It was hypothesized that the C and CF would be the most beneficial to obesity and insulin resistance, however, it was expected that the CF would be more beneficial than the C diet which was not the case. Not only did C group have the lowest fasting serum glucose at termination (Figure 16 d) (which largely affected its HOMA-IR score), it also had one of the lowest fasting serum insulin concentrations (Figure 17 d). This may be intuitive, but, it is important to note that, at week 12, the C also had one of the lowest fasting serum insulin concentrations (Figure 17 b). This indicates that randomization of rats from the original L diet to the treatment diets did not result in similar serum insulin levels at the beginning of the treatment phase; since the C group had the one of the lowest serum insulin concentrations to begin with, it was "easier" for this group to maintain its low insulin levels. Conversely, the CF and SF groups had to experience larger reductions in serum insulin over time in order to end up with some of the lowest HOMA-IR scores which suggest that these diets were, indeed, most beneficial to insulin resistance.

The SF group was not expected to show the beneficial effects that it did. The literature shows that diets high in LA typically exacerbate insulin resistance (Ikemoto *et al*., 1996; Mori *et al*., 1997; Storlein *et al*., 1991). Additionally, correlation analyses showed weak (r^2<0.6) negative associations between dietary MUFAs and serum insulin and between PL MUFAs and serum insulin from rats terminated at week 20 (Table 20). In this same group, PL total PUFAs and n-6 PUFAs were weakly (r^2<0.6) positively associated with serum insulin. The SF group had the lowest MUFA intake (Table 7), the lowest PL MUFA composition (Table16) and among the highest PL total PUFA and n-6 PUFA compositions (Table 16).

A study by Shimomura *et al*. (1990) agrees with the unexpected findings of the current study. They demonstrated lower fasting serum insulin concentration in rats fed a high-fat safflower oil-based diet compared to a lard-based diet, but the safflower group also had a lower percentage of body fat. Furthermore, in the L and LF groups terminated at weeks 12 and 20 in the current study, strong (r^2>0.6) negative correlations were seen between PL n-6/n-3 PUFA ratios and serum glucose. Although this association was not seen in the rats terminated at week 20 and serum glucose was not significantly lower in the SF group at week 20, it may provide some insight as to why the SF group had unexpectedly low HOMA-IR scores; the SF group had the highest n-6/n-3 PUFA ratio among all the treatment groups (Table 18).

Overall, the mechanism by which the SF group in this study had one of the lowest HOMA-IR scores may be explained by a study by Pan *et al*. (1995); this study suggests that skeletal muscle Δ5 desaturase activity, particularly when calculated as

C20:4n6/C20:3n6, is correlated with insulin action and SF group in the current study had the highest C20:4n6/C20:3n6 ratio (Table 18).

ITTs and OGTTs were also used to evaluate insulin resistance. The only statistically significant result was a higher AUC_G in the L-20 group compared to the L-12 group for OGTTs (Table 13). This would indicate a reduction in insulin sensitivity over time, however, no differences were seen in AUC_I and AUC_{GI} between the L-12 and L-20 groups. Therefore, overall, it is difficult to conclude that a change in insulin sensitivity occurred.

It is difficult to explain exactly why significant differences were seen in HOMA-IR scores but not in ITTs and OGTTs, but there are pros and cons for each procedure. For instance, HOMA-IR scores only reflect serum values at a single time point while ITTs and OGTTs measure the body's response to a bolus insulin injection or an oral glucose load over time. Additionally, trunk blood is collected in such a way that both venous and arterial blood are obtained. Other fluids, such as gastric contents could contaminate the sample as well. In particular, if proteolytic enzymes found in the stomach mix with the blood sample, this could have effects on insulin values as insulin in a protein which could be inadvertently be digested. Blood collected from the tail prick method (ITT) and saphenous vein (OGTT) could also be more reliable as it is collected from the extremities; this is more representative of how humans test for blood glucose concentrations (finger prick method).

On the other hand, trunk blood (used for HOMA-IR scores) is collected in a relatively stress free environment after a 12-hour fast; therefore, there are not many external variables present that could confound the results and, perhaps, HOMA-IR scores

are more reliable. Although animals were acclimatized to handling and restraining, ITTs and OGTTs provide more stress to the animal as the animals experience pain from tail pricks (ITTs) or saphenous vein blood collection (OGTTs). A study investigating restraint as a stressor in rats showed increases in serum glucose (Romeo *et al.*, 2007). Additionally, although restrained, the animals have much more movement during ITTs and OGTTs compared to the time surrounding trunk blood collection; it is known that muscle contractions can induce non-insulin stimulated GLUT4 translocation for glucose uptake in muscle tissue (Lauritzen *et al*., 2010), thus, glucose and insulin levels may be influenced by physical activity more than dietary intervention during these tests.

Overall, it is fair to conclude that these dietary interventions did not affect insulin sensitivity. Even though HOMA-IR scores and ITTs/OGTTs provide validated measures of insulin resistance on their own, in practice, a combination of fasting glucose and glucose tolerance are typically used for the diagnosis of prediabetes/DM. It is important to keep in mind that when comparing HOMA-IR scores, ITTs, and OGTTs between weeks 12 and 20 in the L and LF groups, no differences were seen; as stated earlier, insulin resistance is progressive and these results suggest insulin resistance was not adequately established in this model by week 12. Therefore, a longer obesity development phase and/or treatment phase might have yielded different results.

Lipidemia

Fasting serum FFAs did not change over the course of the study in the L and LF groups, nor did they differ at the end of the study among treatment groups (Figures 19 and 20). This is not surprising as the DIO rat literature suggests that serum TGs become

elevated in this model, but there is no mention of serum FFAs (Li *et al*., 2008; Triscari *et al*., 1985). Additionally, a study by Farley *et al*. (2003), showed that fasting serum FFA concentrations did not differ between OR and OP rats. Furthermore, the lack of statistical significance in the FFA data (in the current study) agrees with the insulin resistance results; elevated serum FFAs are known to impair insulin signalling (Belfort *et al*., 2005) and thus, increase insulin resistance.

Fasting serum TGs were clearly elevated from week 12 to week 20 in the L and LF groups (Figure 21 a). Additionally, all treatment groups, except for the HC and CM groups, had lower serum TGs compared to the L group at week 20 and the SB group had the lowest serum TGs among the treatment groups but did not differ from the CF and SF groups (Figure 21 d). It was expected that diets with the higher ALA content would show reductions in serum TGs (Mustad *et al*., 2006). However, the effects seen in the SF and SB groups were, somewhat, unexpected as these diets were high in n-6 PUFAs and a study by Jeffery *et al.* (1996) showed higher serum TGs in rats fed diets with n-6:n-3 ratios greater than 6:1.

One study has shown lower serum TG levels in rats fed safflower oil compared to lard, however, there were no soybean or high-ALA diets in that study (Mori *et al*., 1997). Additionally, a meta-analysis by Mensink and Katan (1992) found that several studies have shown reductions in serum TGs with a high n-6 PUFA diet compared to a high SFA diet. The overall PUFA composition of the diets, rather than n-3 and n-6 PUFAs individually, may explain the findings of the present study. In fact, fasting serum TGs were negatively associated with dietary total PUFAs in addition to dietary n-3 and n-6 PUFAs individually (Table 20). The L group had the lowest amount of total PUFA in the

diet and had the highest fasting serum TGs. Likewise, the SF, followed by the SB, group had the highest total PUFAs in the diet and had the lowest fasting serum TGs. The canola-based groups, fell in the middle for both PUFA and fasting serum TG levels (except for the CF group).

Overall, as mentioned, the C, CF, SF, and SB groups had lower serum TGs compared to the L group at week 20. This effect was seen independent of differences in whole body insulin sensitivity. Because insulin resistance is implicated in increasing hepatic very low density lipoprotein secretion (and, as a result, increasing serum TGs) (Adeli *et al*., 2001) it is proposed here that, perhaps, these diets improved hepatic insulin sensitivity specifically.

Muscle Phospholipid Fatty Acid Composition

In general, the gastrocnemius muscle PL-FA compositions reflected, but did not mimic, the dietary FA compositions. For example, SFA content was highest in the L group and lowest in the canola-based groups (Table 16). This echoes the pattern seen in the dietary FA composition, however, the diets had more extreme variations in SFA content than the PLs; the SFA content of the L diet was approximately 40 g/100 g fatty acids higher than the canola-based groups (Table 7), but the PLs only differed by about 4 g/100 g fatty acids (Table 16). This type of pattern was also seen in the other FA major groupings. The SF group had the highest n-6 PUFA content in their PLs while all the other groups did not differ too greatly from each other in their n-6 PUFA content (Table 16). The CF group, followed by the C group, was highest, while the SF, HC, and L

groups were amongst the lowest, in n-3 PUFA composition. Finally, the canola-based groups showed the highest n-9 MUFA content (Table 16).

These results show that the PLs are affected by dietary FA composition, but are stable in regards to major groupings of FAs. In particular, the PUFA/SFA ratio was not significantly different among groups (Table 18) and this agrees with what is shown in the literature (Andersson *et al*., 2002; Ayre & Hulbert, 1996). Furthermore, the robustness of the PUFA/SFA ratio may explain an adaptation by which these animals preserve insulin sensitivity. This also indicates that the n-6/n-3 ratio of skeletal muscle PLs may not be as important as the PUFA/SFA ratio, in terms of influencing insulin sensitivity, since differences were seen in the n-6/n-3 ratio among groups (Table 18). The reason why the PL-FA composition is so robust is likely because PLs are key components of cell membranes and they need to be able to provide structural support while maintaining a certain degree of membrane fluidity. Typically, SFAs cause more rigid membranes, while long-chain PUFAs provide fluidity (Borkman *et al*., 1993); extremes of either would probably result in dys/non-functional cell membranes.

In keeping with the idea of adaptations, the n-3 PUFAs of interest are quite remarkable. ALA and EPA nearly mirror each other and show large differences among groups (Figure 25 b and d), specifically between the CF and SF groups. One might expect the DHA data to show a similar pattern, however, this is not the case. The DHA values do show differences among groups (Figure 25 f), but compared to the ALA and EPA data, the groups appear to be more consistent in their DHA composition, aside from the SF group which had 4 g/100 g less DHA than the CF group. Additionally, the DHA composition of the PLs is much higher (approximately 8-14 g/100 g FAs) compared to

the ALA and EPA content (trace amounts to approximately 0.9 g/100 g FAs). This suggests that, in the context of the adaptation theory, DHA is the most important n-3 PUFA for maintaining insulin sensitivity. This also indicates that DHA is preferentially incorporated in to skeletal muscle cell membranes over ALA or EPA. It is important to recognize that this does not represent FA use throughout the body so it would be worthwhile to assess FA composition of PLs in other tissues.

It should also be noted that, in this study, ALA was effectively converted to DHA, and to a lesser degree EPA, as reflected by muscle PL-FA composition. Furthermore, this degree of conversion was best when the n-6/n-3 ratio of the diet was 8 or less (Table 7). The SF diet had the lowest amount of DHA in the muscle PLs, but substantial amounts of DHA were present and no detriment to the SF group was seen. This suggests that even very low amounts of dietary ALA can be converted to adequate amounts of DHA. Again, this highlights the importance of DHA in muscle PLs compared to EPA.

Molecular Markers of Insulin Signalling

No differences were seen in the degree of phosphorylation or levels of proteins involved in insulin signalling in gastrocnemius muscle in the present study (Figures 26 and 27). Based on the insulin resistance and PL-FA results, this is not surprising. Additionally, there is not much literature on this topic; of the animals studies cited, both showed non-significant effects of n-3 PUFAs in terms of improving insulin signalling (Le Foll *et al*., 2007; Todoric *et al*., 2006).

It is arguable that, in the fasted state, circulating insulin levels are low and, therefore, insulin signalling proteins are not active in the first place. Thus, measuring

phosphorylation of these proteins is irrelevant. In order to see if this was really the case, Akt was chosen as it is an intermediate in the insulin signalling cascade and can be compared to SAPK/JNK. SAPK/JNK is not an intermediate in the insulin signalling cascade; rather, it acts upon the cascade to inhibit insulin signalling (Lee *et al*., 2003). Phosphorylation of SAPK/JNK is independent of insulin stimulation; it can be activated as a result of chronic inflammation (Lee *et al*., 2003), such as in obesity, therefore, measuring its phosphorylation not only indicates its potential to inhibit insulin signalling, it is also an indirect measure of inflammation. Since differences were not seen in either of the proteins, it can be concluded that the dietary interventions did not have an effect and circulating insulin levels in the fasted state may be adequate to assess insulin signalling protein phosphorylation.

Summary

Overall, it was expected that diets high in ALA would be most beneficial for obesity and its associated consequences, however, this was not observed. Some treatment effects of plant-based dietary oils were seen; all diets appeared to improve triglyceridemia compared to the L diet with the SF diet showing the greatest effect by the end of the study. Additionally, the C, CF, and SF groups had lower HOMA-IR scores compared to the L group at the end of the study. However, overall, insulin sensitivity did not appear to be altered by dietary intervention as indicated by no differences among groups in ITTs, OGTTs, and insulin signalling protein levels and phosphorylation. This may be explained by the robustness of the PUFA/SFA ratio and normalization of DHA in the PLs of gastrocnemius muscle tissue.

XV. SUMMARY

Obesity

- Obesity increased over the course of the study as the L-20 and LF-20 groups had higher body weights, higher body weight/length ratios, more total visceral fat (g/100 g body weight), and less gastrocnemius muscle mass (g/100 g body weight) than the L-12 and LF-12 groups.
- The dietary oil interventions did not affect obesity as body weight, body weight/body length ratios, total visceral fat mass (g/100 g body weight), and gastrocnemius muscle mass (g/100 g body weight) did not differ among treatment groups at the end of the study.

Insulin Resistance

- Fasting serum glucose was not different among groups at week 12 and 16, but the C group had lower fasting serum glucose than all other groups except the SB group at week 20.
- At the end of the study, the C, CF, and SF groups had lower fasting serum insulin compared to the L group.
- The C, CF, and SF groups had lower HOMA-IR scores than the L group at the end of the study.
- No differences were seen among groups for serum glucose concentrations in ITTs.

- Serum glucose and insulin did not differ among groups during OGTTs and for AUC_G, AUC_I, and AUC_{GI}. The only significant difference was an increase in AUC_G between the L-12 and L-20 groups.

Lipidemia

- No differences were seen among groups in fasting serum FFAs.
- Serum TGs increased between the L-20 and LF-20 groups compared to their respective groups at week 12 (L-12 and LF-12, respectively).
- At week 12, the C and LF groups had lower fasting serum TGs than the L group. At week 16 all groups had lower fasting serum TGs than the L group and the SF group was lower than all other groups except the SB group. At week 20, the C, CF, SF and SB groups had lower fasting serum TGs than the L group. The SB group had the lowest fasting serum TGs at week 20, but did not differ from the CF and SF groups.

Muscle Phospholipid Fatty Acid Composition

- Muscle PL-FA composition did not change between the L-12 and L-20 groups nor did it change between the LF-12 and LF-20 groups.
- Major FA groupings in muscle PLs reflected dietary intake to some degree. SFAs were highest in the L, SF, and SB groups; MUFAs were highest in the HC group; PUFAs were highest in the SF groups; n-6 PUFAs were highest in the SF group; n-3 PUFAs were highest in the CF group; n-9 MUFAs were highest in the HC and CM groups.

- Muscle PL-FA ratios followed an expected pattern except the PUFA/SFA was not different among groups.
- Muscle PL ALA and EPA closely reflected dietary ALA composition. However, DHA was more similar among groups except for the SF group which had approximately 4 g/100 g less DHA than the CF group. The SF group had the lowest total n-3 FAs in the muscle PLs.
- A dietary n-6/n-3 ratio of 8 or less appears to allow for the effective conversion of ALA to longer chain n-3 FAs as evidences by the SF group which had the lowest DHA in muscle PLs but still had substantial amounts of this FA. This indicates that small amounts of dietary ALA can be converted to DHA to support muscle DHA concentrations.

Molecular Markers of Insulin Signalling in Muscle

- No differences were seen in levels of Akt or SAPK/JNK among groups.
- No differences were seen in phosphorylation of Akt (at sites Thr^{308} and Ser^{473}) or SAPK/JNK (at sites Thr^{183} and Tyr^{185}) among groups.

Summary

- Dietary oils, may be able to treat certain obesity-associated consequences such as hypertriglyercidemia.
- Low HOMA-IR scores were seen in the C, CF, and SF groups, but other measures of insulin resistance (ITTs, OGTTs, and insulin signalling protein levels and phosphorylation) were not different among groups.

- It appears that this model was able to maintain a consistent response, or adapt, to high-fat diets regardless of the source of fat and this may be attributed to the robustness of the PUFA/SFA ratio and normalization of DHA in gastrocnemius muscle PLs.

XVI. CONCLUSIONS

It was hypothesized that diets containing the highest amounts of n-3 PUFAs (C and CF) would be beneficial, while diets high in n-6 PUFAs (SF and SB) and SFAs (L) would be detrimental to obesity and insulin resistance. Diets high in MUFAs and low in n-3 PUFAs (HC and CM) were expected to be neutral. It was found that, as expected, the C and CF diets were consistently beneficial. However, the SF group (and to a lesser degree, the SB group) also showed benefits. It is difficult to determine if the HC and CM diets had a truly neutral effect as an effective "healthy" control group was not established. The L group did show detrimental effects as it consistently had among the highest values for serum glucose, serum insulin, HOMA-IR scores, and serum TGs at week 20. Table 21 shows a summary of the beneficial effects of various diets.

These results suggest that, in terms of serum glucose, serum insulin, HOMA-IR scores, and serum TGs, the total PUFA content may be more important than the n-3 PUFA content and/or the n-6:n-3 PUFA ratio of high-fat diets. Furthermore, it is important to note that these beneficial effects were observed independently of weight loss or altered body composition and therefore can be directly attributed to the dietary oils.

Other measures used to assess insulin resistance (ITT, OGTT, and insulin signalling protein phosphorylation) did not show differences among groups. This suggests that an adaptation to high-fat feeding, regardless of the source of dietary fat, occurred in the DIO rat model. Possible explanations for this are the robustness of the PUFA/SFA ratio and normalization of DHA in skeletal muscle PLs among groups. It is speculated that a longer obesity development and/or treatment phase may allow for this

adaptation to reach a maximum benefit, after which the true benefits or detriments of the diets could be observed.

Table 21. Summary of the most beneficial diets for various parameters at week 20

	Serum Glucose	Serum Insulin	HOMA-IR	Serum TGs
C	✓	✓	✓	✓ [a]
CF	-	✓	✓	✓ [a,b]
SF	-	✓	✓	✓ [a,b]
SB	-	-	-	✓ [b]

An " ✓ " indicates a lower level as compared to the L group. A "-" indicates no difference as compared to the L group. Superscripts indicate significant differences among groups.

XVII. STRENGTHS

- This study employed a treatment design which is more applicable to pre-developed human obesity.
- The DIO rat model is more representative of human obesity than most other animal models of obesity.
- Multiple dietary oils were used so that comparisons could be made among a variety of dietary FA compositions.
- Plant-based oils were used to investigate the effects of ALA, a dietary FA that is commonly overlooked in obesity research.
- All high-fat diets contained the same amount of energy from fat.
- Obesity and its associated consequences were evaluated in a variety of ways:
    - Obesity - Body weight, body weight/length ratios, visceral fat mass (g/100 g body weight), gastrocnemius muscle mass (g/100 g body weight).
    - Lipidemia - Fasting serum FFAs and TGs.
    - Insulin Resistance - Fasting serum glucose, insulin, and HOMA-IR scores. ITTs, OGTTS, and insulin signalling protein levels and phosphorylation.
- Analyzing gastrocnemius muscle PL-FA composition provided insight into the impact of different dietary oils on tissue FA composition and how this relates to other obesity-associated consequences

XVIII. LIMITATIONS

- An effective control group was not established for this study as the low-fat fed rats were not consistently "healthier" than the high-fat fed rats, and particularly, the L groups.
- High-fat diets contained 55% of energy from fat which is much higher than the AMDR recommendation of 20-35% for humans.
- An insulin stimulation method, just before tissue collection, was not used so insulin signalling protein data reflects response to chronic, but not acute, insulin stimulation.
- HOMA-IR, ITTs, and OGTTs were used as a way to evaluate insulin resistance while the hyperinsulinemic euglycemic clamp remains the gold standard for this assessment.
- A longer obesity development and/or treatment phase may have yielded more significant results.
- Because insulin resistance was not overtly developed, this study did not achieve the treatment design that was desired for investigating this parameter.
- It is assumed that the observed effects of dietary oils were due to the FA composition. Other biologically active components may be present but were not investigated.
- Serum values from blood collected via different procedures (saphenous, jugular, and trunk) and different lengths of fasting cannot be accurately compared.
- A very high ALA diet (for example a flax oil only diet) was not employed for this study.

XIX. FUTURE DIRECTIONS

- Establish an appropriate control group for this model.
- Determine if diets containing various sources of plant-based oils with a lower percent of energy from fat (within the AMDR) would yield similar results.
- Investigate the effects in a longer obesity development and/or treatment phase.
- Determine the biologically active components of the dietary oils.
- Investigate the treatment effects of diets containing very high amounts of ALA.
- Assess insulin resistance by the hyperinsulinemic euglycemic clamp method.
- Collect blood by the same method at different time points to make comparisons over time.

XX. IMPLICATIONS

This study has addressed a number of the limitations of the current research. A DIO rat model was used which better represents human obesity, a variety of oils (particularly canola-based oils and oils high in ALA) were used and contributed the same amount of energy to each diet, and a treatment design was employed. Although treatment effects were seen in some parameters, this study showed that, in general, plant-based sources of n-3 PUFAs are not effective in improving obesity and insulin resistance in a high-fat fed DIO rat model. Additionally, it appears as though an adaptation to high-fat feeding occurs, regardless of the type of dietary fat (HC, C, CM, CF, SF, SB, or L) consumed. Therefore, treatment of obesity and insulin resistance with, for example, dietary fats that are high in ALA may not be effective, but this should be explored and confirmed in other models.

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XXII. APPENDICES

Appendix 1: Weight gain

Weight Gain - week 12-20

	HC	C	CM	CF	SF	SB	L	LF
Weight gain (grams)	52.4 ± 6.3^{c}	$68.8 \pm 9.3^{b,c}$	$81.6 \pm 3.9^{a,b}$	78.1 ± 5.5^{b}	$74.3 \pm 7.4^{b,c}$	$73.9 \pm 6.9^{b,c}$	$88.8 \pm 7.2^{a,b}$	101 ± 10^{a}

Data expressed as mean ± SEM (n=9 or 10 rats/group). Different superscripts indicate significant differences ($p<0.05$) by Duncan's multiple range test. See Table 7 for abbreviations.

Appendix 2: Fat pad mass

A. Week 12 vs. 20

		L-12	L-20	LF-12	LF-20
Visceral adipose[1]	(grams)	45.5 ± 3.3[b]	46.9 ± 3.1[a]	75.8 ± 5.4[b]	63.9 ± 4.6[a]
	(g/100 g bwt)	8.31 ± 0.47[c]	11.45 ± 0.51[a]	8.46 ± 0.41[c]	9.99 ± 0.47[b]
Epididymal adipose	(grams)	15.2 ± 1.1[b]	20.4 ± 1.1[a]	15.3 ± 0.9[b]	17.9 1.0[a,b]
	(g/100 g bwt)	2.78 ± 0.16	3.10 ± 0.10	2.76 ± 0.12	2.82 ± 0.10
Peri-renal adipose	(grams)[2]	21.6 ± 1.5[c]	39.3 ± 3.3[a]	20.8 ± 1.2[c]	29.6 2.3[b]
	(g/100 g bwt)	3.94 ± 0.20[b,c]	5.92 ± 0.33[a]	3.74 ± 0.16[c]	4.63 ± 0.24[b]
Mesenteric adipose	(grams)[3]	8.8 ± 0.9[b]	6.2 ± 3.9[a]	10.9 ± 1.0[b]	16.4 ± 1.6+
	(g/100 g bwt)	1.59 ± 0.14[b]	2.44 ± 0.14[a]	1.95 ± 0.15[b]	2.54 ± 0.20[a]

Data expressed as mean ± SEM (n=10 rats/group). Different superscripts indicate significant differences ($p<0.05$) by Duncan's multiple range test. See Table 7, Table 9 and Figure 6 for abbreviations.

[1]Visceral adipose = sum of epididymal, peri-renal, and mesenteric fat pad masses.

[2]Data were log transformed prior to ANOVA.

[3]Non-parametric testing was used for statistical analyses.

B. Week 20

		HC	C	CM	CF	SF	SB	L	LF
Visceral adipose[1]	(grams)	80.7 ± 7.5	79.9 ± 4.2	82.8 ± 3.6	71.6 ± 3.3	81.3 ± 4.1	74.0 ± 3.2	75.8 ± 5.4	63.9 ± 4.6
	(g/100 g bwt)	12.4 ± 0.8^{a}	12.5 ± 0.4^{a}	12.4 ± 0.4^{a}	11.4 ± 0.4^{a,b}	12.5 ± 0.4^{a}	11.5 ± 0.4^{a,b}	11.5 ± 0.5^{a,b}	9.9 ± 0.5^{b}
Epididymal adipose	(grams)	22.0 ± 1.4^{a}	23.0 ± 0.9^{a}	23.4 ± 0.9^{a}	20.6 ± 0.9^{a,b}	3.4 ± 0.9^{a}	21.5 ± 0.9^{a}	20.4 ± 1.1^{a,b}	17.9 ± 1.0^{b}
	(g/100 g bwt)	3.42 ± 0.13^{a,b}	3.60 ± 0.08^{a}	3.53 ± 0.10^{a}	3.29 ± 0.11^{a,b}	3.62 ± 0.09^{a}	3.34 ± 0.13^{a,b}	3.10 ± 0.10^{b,c}	2.82 ± 0.10^{c}
Peri-renal adipose	(grams)	41.7 ± 5.3	38.2 ± 2.0	39.9 ± 1.6	34.7 ± 1.6	38.8 ± 2.0	34.7 ± 1.4	39.3 ± 3.3	29.6 ± 2.3
	(g/100 g bwt)	5.81 ± 0.25^{a}	5.97 ± 0.23^{a}	6.00 ± 0.17^{a}	5.55 ± 0.20^{a}	5.98 ± 0.22^{a}	5.39 ± 0.19^{a}	5.92 ± 0.33^{a}	4.63 ± 0.24^{b}
Mesenteric adipose	(grams)	17.0 ± 1.3	18.6 ± 1.6	19.5 ± 1.5	16.3 ± 1.1	19.1± 1.3	17.8 ± 1.2	16.2 ± 1.2	16.4 ± 1.6
	(g/100 g bwt)	2.63 ± 0.13	2.89 ± 0.20	2.91 ± 0.18	2.60 ± 0.15	2.94 ± 0.16	2.77 ± 0.17	2.44 ± 0.14	2.54 ± 0.20

Data expressed as mean ± SEM (n=10 rats/group). Different superscripts indicate significant differences ($p<0.05$) by Duncan's multiple range test. See Table 7, Table 9 and Figure 6 for abbreviations.

[1]Visceral adipose = sum of epididymal, peri-renal, and mesenteric fat pad masses.

Appendix 3: Gastrocnemius muscle mass

A. Week 12 vs. week 20

	LF-12	L-20	LF-12	LF-20
Gastrocnemius muscle (grams)	5.79 ± 0.10	5.34 ± 0.22	5.58 ± 0.14	5.14 ± 0.25
Gastrocnemius muscle (g/100 g bwt)[1]	1.07 ± 0.02[a]	0.808 ± 0.042[b]	1.02 ± 0.02[a]	0.818 ± 0.044[b]

Data expressed as mean ± SEM (n=9 or 10 rats per group). Different superscripts indicate significant differences ($p<0.05$) by Duncan's multiple range test. See Table 9 and Figure 6 for abbreviations.
[1]Data were log transformed prior to ANOVA.

C. Week 20

	HC	C	CM	CF	SF	SB	L	LF
Gastrocnemius muscle (grams)[1]	5.00 ± 0.24	5.05 ± 0.26	5.02 ± 0.23	5.03 ± 0.26	5.34 ± 0.18	5.12 ± 0.27	5.34 ± 0.22	5.14 ± 0.25
Gastrocnemius muscle (g/100 g bwt)[1]	0.785 ± 0.039	0.791 ± 0.043	0.760 ± 0.037	0.804 ± 0.037	0.827 ± 0.025	0.795 ± 0.042	0.808 ± 0.042	0.818 ± 0.044

Data expressed as mean ± SEM (n=9 or 10 rats per group). Different superscripts indicate significant differences ($p<0.05$) by Duncan's multiple range test. See Table 7 and Figure 6 for abbreviations.
[1]Data were log transformed prior to ANOVA.

Appendix 4: Serum glucose, serum insulin, HOMA-IR

A. Week 12 vs. week 20

	L-12	L-20	LF-12	LF-20
Glucose (mmol/L)[1]	$16.8 \pm 1.5^{a,b}$	13.9 ± 0.8^{b}	19.4 ± 1.7^{a}	13.8 ± 1.0^{b}
Insulin (nmol/L)	0.235 ± 0.036	0.321 ± 0.045	0.240 ± 0.028	0.330 ± 0.046
HOMA-IR	25.5 ± 5.0	29.2 ± 5.0	36.7 ± 7.4	29.2 ± 4.6

Data expressed as mean ± SEM (n=9 or 10 rats per group). Different superscripts indicate significant differences ($p<0.05$) by Duncan's multiple range test. See Table 9 for abbreviations.
[1]Data were log transformed prior to ANOVA.

B. Fasting serum glucose - weeks 12, 16, and 20

	HC	C	CM	CF	SF	SB	L	LF
Glucose (mmol/L)								
Week 12	11.8 ± 0.8	11.5 ± 0.7	10.8 ± 0.5	10.8 ± 0.6	11.5 ± 0.4	12.4 ± 0.7	11.7 ± 0.5	12.2 ± 1.2
Week 16	11.2 ± 0.4	10.9 ± 0.3	9.67 ± 0.23	11.5 ± 0.6	10.5 ± 0.4	10.3 ± 0.6	11.4 ± 0.8	11.0 ± 0.5
Week 20	12.9 ± 0.7^{a}	10.4 ± 0.7^{b}	13.2 ± 0.7^{a}	12.9 ± 0.6^{a}	13.4 ± 0.7^{a}	$12.2 \pm 0.7^{a,b}$	13.9 ± 0.8^{a}	13.8 ± 1.0^{a}

Data expressed as mean ± SEM (n=10 rats per group). Week 12 and 16 serum was collected from jugular blood after a 5 hour fast, week 20 serum was collected from trunk blood after a 12 hour fast. Different superscripts indicate significant differences ($p<0.05$) by Duncan's multiple range test. See Table 7 for abbreviations.

C. Fasting serum insulin - week 12, 16, and 20

	HC	C	CM	CF	SF	SB	L	LF
Insulin (nmol/L)								
Week 12[1]	0.408 ± 0.050[b]	0.443 ± 0.053[b]	0.516 ± 0.051[a,b]	0.507 ± 0.069[a,b]	0.562 ± 0.056[a,b]	0.703 ± 0.045[a]	0.579 ± 0.104[a,b]	0.412 ± 0.059[b]
Week 16	0.331 ± 0.047	0.307 ± 0.010	0.324 ± 0.040	0.344 ± 0.033	0.403 ± 0.049	0.417 ± 0.075	0.483 ± 0.062	0.446 ± 0.064
Week 20[1]	0.219 ± 0.022[a,b]	0.205 ± 0.021[b]	0.282 ± 0.046[a,b]	0.181 ± 0.015[b]	0.191 ± 0.012[b]	0.250 ± 0.034[a,b]	0.322 ± 0.045[a]	0.329 ± 0.046[a]

Data expressed as mean ± SEM (n=8-10 rats per group). Week 12 and 16 serum was collected from jugular blood after a 5 hour fast, week 20 serum was collected from trunk blood after a 12 hour fast. Different superscripts indicate significant differences ($p<0.05$) by Duncan's multiple range test. See Table 7 for abbreviations.
[1]Data were log transformed prior to ANOVA.

D. HOMA-IR - week 12, 16, and 20

	HC	C	CM	CF	SF	SB	L	LF
HOMA								
Week 12	31.9 ± 5.9	33.8 ± 5.6	35.8 ± 4.0	35.1 ± 5.0	41.9 ± 5.5	57.2 ± 5.1	44.5 ± 9.0	34.0 ± 6.7
Week 16	24.9 ± 4.1	22.1 ± 3.0	20.6 ± 2.5	25.9 ± 3.2	28.7 ± 4.3	29.3 ± 6.2	36.2 ± 5.2	33.3 ± 5.5
Week 20[1]	18.2 ± 2.0[a,b,c]	13.7 ± 1.8[c]	23.1 ± 3.0[a,b]	15.5 ± 4.6[b,c]	16.5 ± 1.5[b,c]	20.2 ± 3.5[a,b,c]	29.3 ± 5.0[a]	29.3 ± 4.5[a]

Data expressed as mean ± SEM (n=8-10 rats per group). Week 12 and 16 serum was collected from jugular blood after a 5 hour fast, week 20 serum was collected from trunk blood after a 12 hour fast. Different superscripts indicate significant differences ($p<0.05$) by Duncan's multiple range test. See Table 7 for abbreviations.
[1]Data were log transformed prior to ANOVA.

Appendix 5: Serum free fatty acids and triglycerides

A. Fasting serum free fatty acids - week 12 vs. week 20

	L-12	L-20	LF-12	LF-20
Triglycerides (mmol/L)[1]	2.09 ± 0.35[b]	5.97 ± 1.07[a]	2.95 ± 0.54[b]	4.99 ± 0.59[a]
Free fatty acids (mmol/L)	0.323 ± 0.026	0.384 ± 0.014	0.376 ± 0.037	0.416 ± 0.047

Data expressed as mean ± SEM (n=9 or 10 rats per group). Different superscripts indicate significant differences ($p<0.05$) by Duncan's multiple range test. See Table 9 for abbreviations.
[1]Data were log transformed prior to ANOVA.

B. Fasting serum free fatty acids - week 20

	HC	C	CM	CF	SF	SB	L	LF
Free fatty acids (mmol/L)	0.396 ± 0.019	0.380 ± 0.038	0.370 ± 0.032	0.443 ± 0.031	0.381 ± 0.017	0.372 ± 0.030	0.384 ± 0.014	0.416 ± 0.047

Data expressed as mean ± SEM (n=10 rats per group). An absence of superscripts indicates no significant differences. See Table 7 for abbreviations.

C. Fasting Serum Triglycerides - Weeks 12, 16, and 20

	HC	C	CM	CF	SF	SB	L	LF
Triglycerides (mmol/L)								
Week 12	6.17 ± 0.66[a]	4.19 ± 0.42[b,c]	5.58 ± 0.53[a,b]	5.42 ± 0.54[a,b]	5.47 ± 0.59[a,b]	5.38 ± 0.46[a,b]	6.39 ± 0.64[a]	3.76 ± 0.26[c]
Week 16[1]	4.85 ± 0.59[b]	3.53 ± 0.49[b,c]	4.46 ± 0.57[b]	3.58 ± 0.46[b,c]	2.07 ± 0.32[d]	2.53 ± 0.32[c,d]	7.57 ± 0.71[a]	4.09 ± 0.49[b]
Week 20[1]	5.26 ± 0.73[a,b]	3.66 ± 0.31[b,c]	4.04 ± 0.42[a,b,c]	2.81 ± 0.33[d,c]	2.87 ± 0.41[c,d]	2.50 ± 0.30[d]	5.97 ± 1.07[a]	4.99 ± 0.59[a,b]

Data expressed as mean ± SEM (n=9-10 rats per group). Week 12 and 16 serum was from jugular blood, week 20 serum was from trunk blood. Different superscripts indicate significant differences ($p<0.05$) by Duncan's multiple range test. See Table 7 for abbreviations.

[1]Data were log transformed prior to ANOVA.

Appendix 6: Gastrocnemius muscle phospholipid fatty acid composition

A. Week 12 vs. Week 20

Fatty Acids (g/100 g FAs)	L-12	L-20	LF-12	LF-20
C8:0	0.955 ± 0.172	0.740 ± 0.221	0.718 ± 0.192	0.462 ± 0.123
C10:0	0.208 ± 0.078	0.288 ± 0.024	0.247 ± 0.069	0.317 ± 0.049
C12:0	0.206 ± 0.037	0.469 ± 0.181	0.419 ± 0.157	0.276 ± 0.110
C14:0	0.398 ± 0.060	0.375 ± 0.042	0.269 ± 0.024	0.282 ± 0.017
C14:1	0.141 ± 0.039	0.267 ± 0.164	0.056 ± 0.035	0.240 ± 0.022
C15:1	1.15 ± 0.36	1.00 ± 0.48	0.357 ± 0.057	0.890 ± 0.38
C16:0	18.5 ± 1.1[b]	20.1 ± 0.8[a,b]	21.8 ± 0.3[a]	21.0 ± 0.5[a]
C16:1n9	0.724 ± 0.125	0.643 ± 0.053	0.547 ±0.035	0.637 ± 0.023
C16:1n7	0.550 ± 0.123	0.335 ± 0.088	0.0736 ± 0.0467	0.182 ± 0.052
C17:0	1.14 ± 0.34	0.915 ± 0.069	0.530 ± 0.022	0.589 ± 0.031
C18:0	19.4 ± 0.8	17.7 ± 0.4	16.0 ± 0.6	15.6 ± 0.3
C18:1n9[1]	5.70 ± 0.46[a]	6.46 ± 0.43[a]	3.58 ± 0.15[b]	3.50 ± 0.17[b]
C18:1n7[2]	1.67 ± 0.40[b,c]	1.57 ± 0.37[c]	2.63 ± 0.09[a]	2.45 ± 0.05[a,b]
C18:2n6	14.5 ± 0.7[b]	13.5 ± 1.3[b]	18.7 ± 0.7[a]	19.7 ± 0.8[a]
C18:3n3	0.228 ± 0.062[b]	0.254 ± 0.071[b]	0.429 ± 0.043[a]	0.531 ± 0.021[a]

C20:0	0.192 ± 0.021	0.197 ± 0.031	0.140 ± 0.038	0.134 ± 0.014
C20:1	0.355 ± 0.060[a]	0.307 ± 0.020[a]	0.123 ± 0.056[b]	0.194 ± 0.064[a,b]
C20:2n6	1.83 ± 0.16	1.47 ± 0.30	1.40 ± 0.05	1.23 ± 0.08
C20:3n6[1]	1.14 ± 0.07[a]	1.22 ± 0.07[a]	0.89 ± 0.03[b]	0.90 ± 0.02[b]
C20:4n6	14.9 ± 0.9	15.8 ± 0.5	13.2 ± 0.6	13.8 ± 0.5
C20:3n3	0.160 ± 0.028	0.102 ± 0.027	0.071 ± 0.048	0.172 ± 0.034
C20:5n3	0.158 ± 0.022	0.137 ± 0.005	0.208 ± 0.021	0.169 ± 0.020
C22:0	0.331 ± 0.068	0.404 ± 0.115	0.318 ± 0.036	0.212 ± 0.036
C22:4n6	0.539 ± 0.025[a]	0.530 ± 0.016[a]	0.478 ± 0.032[a,b]	0.418 ± 0.010[b]
C22:6n3	12.0 ± 0.6[b]	12.3 ± 0.4[b]	13.8 ± 0.2[a]	12.7 ± 0.3[a,b]
C24:0	0.503 ± 0.077	0.566 ± 0.122	0.499 ± 0.049	0.396 ± 0.050
C24:1[2]	0.197 ± 0.056	0.328 ± 0.077	0.088 ± 0.055	0.084 ± 0.053

Data expressed as mean ± SEM (n=5 rats per group). Different superscripts indicate significant differences ($p<0.05$) by Duncan's multiple range test. See Table 9 for abbreviations.

[1]Data were log transformed prior to ANOVA.

[2]Non-parametric testing was used for statistical analyses.

B. Week 20

Fatty Acids (g/100 g FAs)	HC	C	CM	CF	SF	SB	L	LF
C8:0	0.476 ± 0.065	0.579 ± 0.081	0.594 ± 0.090	0.711 ± 0.153	0.494 ± 0.086	0.541 ± 0.174	0.740 ± 0.221	0.462 ± 0.123
C10:0	0.189 ± 0.022	0.288 ± 0.052	0.321 ± 0.074	0.254 ± 0.044	0.352 ± 0.062	0.334 ± 0.059	0.288 ± 0.024	0.317 ± 0.049
C12:0	0.256 ± 0.064	0.159 ± 0.050	0.288 ± 0.099	0.415 ± 0.092	0.574 ± 0.205	0.143 ± 0.030	0.469 ± 0.181	0.276 ± 0.110
C14:0	0.233 ± 0.026	0.261 ± 0.015	0.287 ± 0.027	0.248 ± 0.028	0.230 ± 0.026	0.398 ± 0.127	0.375 ± 0.042	0.282± 0.017
C14:1	0.128 ± 0.040	0.275 ± 0.062	0.185 ± 0.062	0.075 ± 0.049	0.200 ± 0.015	0.575 ± 0.447	0.267 ± 0.164	0.240 ± 0.0221
C16:0	18.4 ± 0.3^{b}	18.8 ± 0.8^{b}	$20.1 \pm 0.7^{a,b}$	18.7 ± 0.6^{b}	$20.0 \pm 0.8^{a,b}$	21.1 ± 0.8^{a}	$20.1 \pm 0.8^{a,b}$	21.2 ± 0.4^{a}
C16:1n9	0.698 ± 0.042	0.611 ± 0.109	0.623 ± 0.105	0.580 ± 0.087	0.370 ± 0.043	0.449 ± 0.070	0.643 ± 0.053	0.637 ± 0.023
C16:1n7	0.350 ± 0.073	0.383 ± 0.163	0.334 ± 0.020	0.289 ± 0.057	0.191 ± 0.054	0.244 ± 0.071	0.335 ± 0.088	0.182 ± 0.052
C17:0	0.688 ± 0.113	1.058 ± 0.482	0.663 ± 0.045	0.528 ± 0.026	0.490 ± 0.138	0.657 ± 0.027	0.915 ± 0.069	0.589 ± 0.031
C17:1	0.223 ± 0.067	0.398 ± 0.262	0.187 ± 0.032	0.117 ± 0.034	0.116 ± 0.041	0.163 ± 0.015	0.248 ± 0.028	0.115 ± 0.014
C18:0	15.5 ± 0.3^{c}	16.3 ± 0.6^{c}	15.4 ± 0.5^{c}	15.9 ± 0.4^{c}	18.4 ± 0.3^{a}	$16.5 \pm 0.6^{b,c}$	$17.7 \pm 0.4^{a,b}$	15.6 ± 0.3^{c}
C18:1n9[1]	9.86 ± 0.14^{a}	$7.93 \pm 0.24^{a,b}$	9.08 ± 0.88^{a}	$8.50 \pm 0.50^{a,b}$	3.51 ± 0.19^{c}	6.38 ± 1.96^{b}	5.46 ± 0.43^{b}	3.50 ± 0.17^{c}
C18:1n7	2.44 ± 0.59	2.79 ± 0.16	2.28 ± 0.56	2.04 ± 0.49	1.73 ± 0.07	1.93 ± 0.07	1.57 ± 0.37	2.45 ± 0.05
C18:2n6	$15.1 \pm 0.8^{d,c}$	$15.4 \pm 0.3^{d,c}$	$14.4 \pm 0.6^{d,c}$	$17.2 \pm 0.6^{b,c}$	20.3 ± 0.8^{a}	$16.9 \pm 1.5^{b,c}$	13.5 ± 1.3^{d}	$19.7 \pm 0.8^{a,b}$
C18:3n3	$0.220 \pm 0.019^{e,d}$	$0.473 \pm 0.046^{c,d}$	$0.343 \pm 0.063^{c,d}$	0.861 ± 0.031^{a}	0.135 ± 0.060^{e}	$0.455 \pm 0.026^{c,b}$	$0.254 \pm 0.071^{e,d}$	0.531 ± 0.021^{b}
C20:0	0.220 ± 0.021	0.193 ± 0.018	0.277 ± 0.058	0.230± 0.068	0.175 ± 0.009	0.203 ± 0.046	0.197 ± 0.031	0.134 ± 0.014

C20:1[2]	0.361 ± 0.015[a]	0.342 ± 0.027[a]	0.360 ± 0.018[a]	0.269 ± 0.068[a,b]	0.119 ± 0.049[a,b]	0.291 ± 0.031[a,b]	0.307 ± 0.020[a,b]	0.194 ± 0.064[b,c]
C20:2n6	1.30 ± 0.06	1.33 ± 0.06	1.19 ± 0.29	1.46 ± 0.10	1.80 ± 0.16	1.67 ± 0.18	1.47 ± 0.30	1.26 ± 0.09
C20:3n6[1]	1.21 ± 0.03[ab]	1.08 ± 0.04[b]	1.24 ± 0.07[a,b]	1.27 ± 0.02[a]	0.85± 0.02[c]	0.89± 0.09[c]	1.22 ± 0.07[a,b]	0.90 ± 0.02[c]
C20:4n6	16.2 ± 0.6[a,b]	12.9 ± 0.7[c,d]	13.6 ± 0.5[c]	11.2 ± 0.5[d]	17.7 ± 0.7[a]	12.5 ± 0.8[c,d]	15.8 ± 0.5[b]	13.8 ± 0.5[c]
C20:3n3	0.0998 ± 0.0267	0.141 ± 0.022	0.129 ± 0.012	0.116 ± 0.030	0.0504 ± 0.0208	0.134 ± 0.014	0.102 ± 0.027	0.172 ± 0.034
C20:5n3	0.113 ± 0.006[d]	0.284 ± 0.016[b]	0.190 ± 0.018[c]	0.549 ± 0.046[a]	0.000 ± 0.000[e]	0.161 ± 0.021[c,d]	0.137 ± 0.005[c,d]	0.169 ± 0.020[c,d]
C22:0	0.327 ± 0.065	0.229 ± 0.031	0.199 ± 0.186	0.492 ± 0.178	0.229 ± 0.022	0.444 ± 0.174	0.404 ± 0.115	0.212 ± 0.036
C22:4n6[2]	0.516 ± 0.015[b]	0.286 ± 0.009[d]	0.382 ± 0.024[c]	0.187 0.052[e]	1.24 ± 0.05[a]	0.363 ± 0.018[c,d]	0.530 ± 0.015[b]	0.418 ± 0.010[c]
C22:5n3	1.39 ± 0.12[e]	1.98 ± 0.14[c]	1.85 ± 0.11[c,d]	2.94 ± 0.09[a]	0.67 ± 0.03[f]	1.72 ± 0.12[c,d,e]	1.64 ± 0.09[d,e]	2.43 ± 0.12[b]
C22:6n3[1]	12.2 ± 0.1[c]	13.6 ± 0.4[a]	13.2 ± 0.2[a,b]	13.0 ± 0.3[a,b,c]	9.03 ± 0.38[d]	12.5 ± 0.4[b,c]	12.3 ± 0.4[c]	12.7 ± 0.3[b,c]
C24:0	0.418 ± 0.060	0.374 ± 0.042	0.620 ± 0.149	0.691 ± 0.160	0.371 ± 0.021	0.678 ± 0.172	0.566 ± 0.122	0.396 ± 0.050
C24:1	0.332 ± 0.036	0.241 ± 0.026	0.441 ± 0.132	0.378 ± 0.124	0.121 ± 0.051	0.359 ± 0.155	0.328 ± 0.0774	0.084 ± 0.0526

Data expressed as mean ± SEM (n=5 rats per group). Different superscripts indicate significant differences ($p<0.05$) by Duncan's multiple range test. See Table 7 for abbreviations.

[1]Data were log transformed prior to ANOVA.

[2]Non-parametric testing was used for statistical analyses.

Appendix 7. Relative insulin signalling protein phosphorylation and levels in gastrocnemius muscle

		HC	C	CM	CF	SF	SB	L	LF	L-12	LF-12
pAkt Thr^{308}											
	phosphorylation	0.944 ± 0.351	1.51 ± 0.66	1.11 ± 0.36	1.75 ± 1.20	0.933 ± 0.323	1.93 ± 1.27	2.52 ± 1.37	1.21 ± 0.58	0.576 ± 0.098	0.739 ± 0.221
	protein levels	1.77 ± 0.60	1.45 ± 0.53	2.18 ± 0.80	2.21 ± 0.90	1.86 ± 0.61	2.43 ± 0.66	2.29 ± 0.65	3.64 ± 1.18	1.71 ± 0.67	1.98 ± 0.83
pAkt Ser^{473}											
	phosphorylation	0.790 ± 0.305	0.611 ± 0.266	1.19 ± 0.34	1.08 ± 0.30	0.830 ± 0.330	1.10 ± 0.32	1.07 ± 0.28	0.943 ± 0.294	0.867 ± 0.345	1.02 ± 0.24
	protein levels	2.13 ± 0.92	2.83 ± 1.79	2.26 ± 1.54	1.67 ± 1.00	2.33 ± 1.58	1.47 ± 0.72	1.57 ± 0.82	2.10 ± 1.51	1.32 ± 0.36	0.826 ± 0.187
pSAPK/JNK Thr^{183}/Tyr^{185}											
	phosphorylation	1.99 ± 1.04	2.57 ± 1.18	2.22 ± 1.13	2.73 ± 1.09	2.44 ± 1.25	3.15 ± 1.18	3.23 ± 1.12	3.85 ± 1.39	1.38 ± 0.39	1.84 ± 0.81
	protein levels	0.886 ± 0.212	1.13 ± 0.25	1.39 ± 0.31	1.25 ± 0.17	1.48 ± 0.46	1.05 ± 0.18	0.949 ± 0.114	0.901 ± 0.117	0.816 ± 0.173	0.894 ± 0.278

All data expressed as mean ± SEM (n=4 rats/group) in arbitrary units. All data was adjusted to control for differences in band intensities on different membranes. Phosphorylation and protein levels were adjusted to control for differences in protein loading. An absence of superscripts indicates no significant differences. There were no significant differences when week 20 and week 12 vs. 20 statistics were run separately. See Tables 7 and 9 for abbreviations. Thr^{308}, phosphorylated at threonine-308; Ser^{473}, phosphorylated at serine-473; Thr^{183}, phosphorylated at threonine-185; Tyr^{185}, phosphorylated tyrosine-185.

Appendix 8. All correlations

Diet fatty acid composition vs. serum parameters

Time	Parameter 1	Parameter 2	r^2	p-value
Week. 20	diet SFA	serum TGs	0.325	0.003
		serum glucose	0.196	0.082
		serum insulin	0.292	0.011
		HOMA-IR	0.354	0.001
	diet MUFA	serum TGs	0.157	0.164
		serum glucose	-0.149	0.188
		serum insulin	-0.269	0.019
		HOMA-IR	-0.151	0.1883
	diet PUFA	serum TGs	-0.325	0.003
		serum glucose	0.035	0.7577
		serum insulin	0.094	0.422
		HOMA-IR	-0.045	0.697
	diet n-6 PUFA	serum TGs	-0.255	0.024
		serum glucose	0.063	0.579
		serum insulin	0.118	0.312
		HOMA-IR	-0.014	0.902
	diet n-3 PUFA	serum TGs	-0.253	0.025
		serum glucose	-0.102	0.368
		serum insulin	-0.092	0.432
		HOMA-IR	-0.125	0.274
	diet n6/n3 PUFA ratio	serum TGs	-0.211	0.062
		serum glucose	0.086	0.450
		serum insulin	0.043	0.712
		HOMA-IR	-0.146	0.204

All data analyzed using Pearson's Correlation. Values are considered significant when $p<0.05$. n=75 to 80, all groups terminated at week 20.

Phospholipid fatty acid composition vs. serum parameters

Time	Parameter 1	Parameter 2	r^2	p-value
Weeks 12 vs. 20[1]	PL SFA	serum TGs	-0.008	0.974
		serum glucose	-0.155	0.514
		serum insulin	0.079	0.749
		HOMA-IR	0.353	0.127
	PL MUFA	serum TGs	-0.016	0.951
		serum glucose	-0.463	0.053
		serum insulin	0.229	0.376
		HOMA-IR	-0.189	0.454
	PL PUFA	serum TGs	0.186	0.445
		serum glucose	0.235	0.318
		serum insulin	-0.194	0.425
		HOMA-IR	-0.168	0.478
	PL n-6 PUFA	serum TGs	0.139	0.571
		serum glucose	0.009	0.970
		serum insulin	-0.098	0.690
		HOMA-IR	-0.158	0.507
	PL n-3 PUFA	serum TGs	0.191	0.433
		serum glucose	0.555	0.011
		serum insulin	-0.301	0.210
		HOMA-IR	-0.123	0.606
	PL n6/n3 PUFA ratio	serum TGs	-0.141	0.565
		serum glucose	-0.603	0.005
		serum insulin	0.255	0.292
		HOMA-IR	-0.021	0.930

Week. 20[2]	PL SFA	serum TGs	-0.107	0.516
		serum glucose	0.280	0.081
		serum insulin	0.160	0.353
		HOMA-IR	0.211	0.191
	PL MUFA	serum TGs	0.013	0.936
		serum glucose	-0.169	0.297
		serum insulin	-0.393	0.018
		HOMA-IR	0.125	0.441
	PL PUFA	serum TGs	0.096	0.562
		serum glucose	0.005	0.977
		serum insulin	0.369	0.027
		HOMA-IR	-0.253	0.116
	PL n-6 PUFA	serum TGs	0.084	0.613
		serum glucose	0.140	0.389
		serum insulin	0.343	0.041
		HOMA-IR	-0.173	0.286
	PL n-3 PUFA	serum TGs	-0.019	0.908
		serum glucose	-0.254	0.113
		serum insulin	-0.086	0.618
		HOMA-IR	-0.057	0.725
	PL n6/n3 PUFA ratio	serum TGs	0.001	0.994
		serum glucose	0.271	0.091
		serum insulin	0.172	0.317
		HOMA-IR	-0.088	0.590

All data analyzed using Pearson's Correlation. Values are considered significant when $p<0.05$.
[1]n=20, lard and low-fat groups terminated at weeks 12 and 20.
[2]n=36 to 40, all groups terminated at week 20.

Appendix 9: Comparison of serum glucose collected from saphenous, jugular, and trunk blood

	Saphenous[1]	Jugular[1]	Trunk[2]
Serum glucose (mmol/L)	7.68 ± 0.32^{c}	11.5 ± 0.8^{b}	14.4 ± 0.8^{a}

Data expressed as mean ± SEM (n=10 rats per group, same rats were used for each collection method). Different superscripts indicate significant differences ($p<0.05$) by Duncan's multiple range test.
[1]Blood collected after 5 hour fast; saphenous collected immediately before jugular.
[2]Blood collected after 12 hour fast on the morning of the day following saphenous and jugular blood collection.

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