THE EFFECTS OF DIETARY ZINC OR n-3 FATTY ACIDS ON HYPERINSULINEMIA, HYPERLIPIDEMIA, AND PANCREATIC FUNCTION IN fa/fa AND LEAN ZUCKER RATS

By

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A thesis submitted to the Department of Human Nutritional Sciences in partial fulfillment of the requirements for the degree of Master of Science

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FACULTY OF GRADUATE STUDIES

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BY

Melani E. Gillam

A Thesis/Practicum submitted to the Faculty of Graduate Studies of The University of Manitoba in partial fulfillment of the requirement of the degree

Of

MASTER OF SCIENCE

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ABSTRACT

THE EFFECTS OF DIETARY ZINC OR n-3 FATTY ACIDS ON HYPERINSULINEMIA, HYPERLIPIDEMIA, AND PANCREATIC FUNCTION IN fa/fa AND LEAN ZUCKER RATS

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Sub-optimal zinc status and impaired glucose and lipid metabolism have been implicated in development of obesity and Type 2 diabetes (DM2); however, research is limited on the effects of altered dietary zinc or fatty acids on insulin resistance, lipid metabolism (hyperlipidemia) and pancreatic function. Therefore, the objectives of this thesis were to compare the effects of dietary zinc (Zinc Study), and dietary n-6, long (C18) or very long (≥ C20) chain n-3 fatty acids (Lipid Study) on hyperinsulinemia, hyperlipidemia and pancreatic function in fa/fa (fa) Zucker rats, a genetic model of obesity and insulin resistance.

Five-week-old male fa and lean (ln) Zucker rats were used for the 9-week Zinc (n=56) and Lipid (n=64) Studies. Zinc Study diets were zinc deficient (ZD, 5 ppm zinc), zinc control (ZC, 30 ppm zinc), or zinc supplemented (ZS, 150 ppm zinc). Lipid Study diets had 10% (w/w) dietary oil mixtures containing primarily flaxseed oil (FXO), menhaden oil (MO) or safflower oil (SO) and similar amounts of saturated (SAT), monounsaturated (MUFA) and polyunsaturated (PUFA) fatty acids. At week 8.5, only Lipid Study rats underwent an oral glucose tolerance test (OGTT). For both Studies, fasting serum was analyzed for C-peptide, triglycerides, free fatty acids (FFA), leptin and

zinc concentrations, while pancreas was assessed for insulin and metallothionein (MT) immunolocalization and zinc concentration.

The fa rats had higher body, epididymal and perirenal fat pad weights, lower pancreas weights, as well as higher fasting serum insulin concentrations than ln Zucker rats. Dietary oil mixtures did not influence these parameters, except fasting serum insulin concentrations were lowest in the SO rats. The fa rats of both Studies had higher fasting serum-C-peptide, leptin, triglyceride and FFA concentrations compared to ln Zucker rats. Both dietary zinc and oil mixtures did not influence serum parameters, except serum FFA concentrations were lower in MO than FXO and SO rats and serum zinc concentrations were higher in SO rats. Zinc Study ln and fa ZD Zucker rats had lower pancreas zinc concentrations compared to ZC and ZS rats, but no dietary effect was observed in the Lipid Study. Lipid Study fa rats had impaired glucose tolerance at t=15 and t=30 minutes of an OGTT compared to ln Zucker rats. Qualitatively, the fa rats had more islets (insulin immunostaining) compared to lean rats; however, there were no dietary effects in both Studies. Qualitatively, more intense MT staining was observed in fa than ln Zucker rats of both Studies. In conclusion, dietary zinc, or moderate (10%w/w) fat diets with controlled SAT, MUFA, PUFA amounts, but different n-6 and n-3 fatty acid compositions, did not influence obesity, hyperinsulinemia and pancreatic function in the fa Zucker rat. It appears that the potential benefits of dietary PUFA interventions for insulin sensitivity may not be attributed to certain PUFA but rather to the PUFA/SAT ratio.

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To the special boys in my life, Blaine and Leslie, I dedicate this Master's thesis to you; it is as much yours as it is mine. I LOVE YOU!

A special dedication is also given to my wonderful father, Leslie Edward Little. Thank you for your prayers and companionship.

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LIST OF ABBREVIATIONS

AA = Arachidonic Acid

ALA = α-Linolenic Acid

B_0 = Reference

BSA = Bovine Serum Albumin

CPM = Counts Per Minute

DAB = Diaminobenzidine Tetrahydrochloride

DHA = Docosahexaenoic Acid

DIO = Diet-induced Obesity

DM = Diabetes Mellitus

DM2 = Type 2 DM

EPA = Eicosapentaenoic Acid

fa = Fatty

fa/fa = Fatty

faFXO = fa/fa Zucker rat fed flaxseed oil diet

faMO = fa/fa Zucker rat fed menhaden oil diet

faSO = fa/fa Zucker rat fed safflower oil diet

FFA = Free Fatty Acid

FXO = Flaxseed Oil Mixture

GSIS = Glucose-stimulated Insulin Secretion

HPLC = High Performance Liquid Chromatography

LA = Linoleic Acid

ln = Lean

lnFXO = Lean rat fed flaxseed oil diet

lnMO = Lean rat fed menhaden oil diet

lnSO = Lean rat fed safflower oil diet

MO = Menhaden Oil mixture

MT = Metallothionein

MUFA = Monounsaturated Fatty Acid

Ob-R = Leptin Receptor

OGTT = Oral Glucose Tolerance Test

PBS = Phosphate Buffered Solution

ppm = Parts Per Million

PUFA = Polyunsaturated Fatty Acid

PW = Pair-weighed

RIA = Radioimmunoassay

SAT = Saturated Fatty Acid

SO = Safflower Oil

STZ = Streptozotocin

VLDL = Very Low Density Lipoprotein

w/w = Weight/weight

ZC = Zinc-control

ZD = Zinc-deficient

ZS = Zinc-Supplement

I. LITERATURE REVIEW

INTRODUCTION

Canada is currently experiencing an aging population, with increasing incidence and prevalence of diabetes mellitus, particularly Type 2 diabetes mellitus (DM2). The usual age range for the onset of this disease is between 45-50 years of age (Holler and Green Pastors 1997). This age range coincides with the largest section of the Canadian population, the Baby Boomers (born between 1946-1962) that comprise almost 33% of the population (Foot 1996). Increasing incidence and prevalence rates of DM2 are also observed in Canada's Aboriginal population. For example, DM prevalence rates in 1991 for Manitoba's Treaty-Indian men and women were 12% and 20%, respectively (Diabetes Burden of Illness Study 1995). Increasing incidence and prevalence rates in Canada's aging population, as well as in Canada's Aboriginal population predict the beginnings of a "coming storm" of DM2 that may manifest itself through greatly increased health care costs and the loss of quality of life for many people. Therefore, research, such as basic nutrition research, to understand biological mechanisms of DM2 will form part of the foundation for developing new prevention and treatment approaches to lower the severity and hopefully obliterate this storm.

Obesity (especially central obesity) and insulin resistance are key risk factors of DM2 (Knowler 1995, Kahn 1997 and reviewed by Solomon and Manson 1997). Several nutrients have been implicated in the development of insulin resistance (Mooradian et al. 1994). Research has indicated hyperzincuria (Walter et al. 1991 and Taneja et al.1998), possible lower zinc status (Walter et al. 1991, and Blostein-Fuji et al. 1997), as well as

abnormal glucose and lipid metabolisms in both obesity and DM2 (DeFronzo 1992, Unger 1995, and Grundy 1998). Dietary zinc supplementation studies show improved zinc status in some DM2 animal models (Simon 1998, and Tobia et al. 1998), but results of similar research in obese animals is lacking. Improved peripheral glucose uptake with lowered blood lipid concentrations have been documented in diet-induced obesity studies using animals fed high fat diets made with fish oils (Storlein et al. 1987, Topping et al. 1987, Storlein et al. 1991, and Luo et al. 1996). However, research is limited on the effects of fish oils (n-3 fatty acids) on similar parameters in genetically obese animals. In addition, few studies have investigated the effects of dietary oils or dietary zinc on pancreatic function, the other piece of the puzzle in insulin resistance and DM2. Therefore, the purpose of this thesis is to investigate the effects of dietary zinc or n-3 fatty acids on hyperlipidemia and pancreatic function in the fa/fa Zucker rat, an animal model of obesity and insulin resistance. This literature review will present current knowledge for these topic areas.

DIABETES MELLITUS

Diabetes mellitus (DM) is a chronic disease in which there is impaired carbohydrate, protein and lipid metabolism (Hurwitz and Porth 1986). The diagnostic criteria used for DM are symptoms of DM (e.g. excessive thirst, polyuria) plus a causal plasma glucose concentration of 11.1 mmol/L or more, or a fasting plasma glucose concentration greater than 7.0 mmol/L (Meltzer et al. 1998). Normal blood glucose homeostasis is lost. Impaired glucose control could result from insufficient or no insulin production, or from peripheral resistance to insulin (Chausmer 1998). Insulin is a

hormone produced by the pancreas to help the body control and maintain blood glucose concentrations, lipid and protein metabolisms.

There are two types of DM. Type 1 DM or juvenile diabetes is the result of very little or no insulin production by the pancreas. The prevalence of Type 1 DM is highest in people under the age of thirty where it usually occurs in children, adolescents and young adults (Holler and Green Pastors 1997). Type 1 DM is an autoimmune disease in which pancreatic β cells are destroyed, resulting in insufficient synthesis of insulin (Sprietsma and Schuitemaker 1993). People with Type 1 DM can be subjected to wide variations of blood glucose concentrations from extreme hyperglycemia to hypoglycemia where diabetic coma may result. People with Type 1 DM are also prone to ketoacidosis, which is a condition characterized by very high levels of blood glucose and ketone bodies (Holly and Green Pastors 1997). Insulin therapy is required to help control and maintain normal blood glucose levels and prevent excess formation of ketone bodies (Chausmer 1998).

Type 2 DM, which is the focus of this study, occurs when there is insufficient pancreatic insulin production and/or peripheral insulin resistance. Initial stages of Type 2 DM begin with resistance of target organs (liver, skeletal muscle, adipose tissue) to insulin. Resistance to insulin results in impaired glucose uptake and hyperglycemia. Elevated blood glucose concentrations prompt an increase in insulin synthesis and secretion by pancreatic β cells, thus causing hyperinsulinemia and down-regulation of insulin receptors on the target tissues (Chausmer 1998). As Type 2 DM progresses, β-cell exhaustion eventually results in insufficient insulin production to control blood glucose levels (Chausmer 1998). Even with insufficient insulin synthesis and secretion,

people with Type 2 DM are not usually prone to ketoacidosis as they secrete sufficient insulin amounts to prevent significant ketone synthesis (Holly and Green Pastors 1997).

Although observed in children and young adults (Chausmer 1998), Type 2 DM is more common in people greater than 40 years old (Holler and Green Pastors 1997). Because people diagnosed with Type 2 DM are at various stages of pancreatic function and insulin resistance, there are different treatments. These treatments range from dietary control and lifestyle changes, hypoglycemic agents such as sulfonylureas, and insulin to help control and maintain normal blood glucose concentrations (Chausmer 1998). Chronically, uncontrolled blood glucose concentrations can lead to serious complications such as neuropathy, blindness, kidney failure, limb amputation, cardiovascular disease, and eventually death (Holler and Green Pastors 1997).

Incidence and Prevalence of Type 2 Diabetes

Although the incidence rate of Type 2 DM has gradually decreased in both male and females in Manitoba in the $\geq$25 year olds (7.5 to 6.0/1000 and 6.8 to 5.3/1000, respectively), the prevalence of type 2 diabetes mellitus in Manitoba has risen steadily in both men and women to the 1991 level of 66.9/1000 (Blanchard et al. 1996). Both the incidence and prevalence of Type 2 DM increase with age. According to Blanchard and colleagues (1996), the incidence of DM increased from 0.9/1000 in men aged 25-29 years to 16.7/1000 in men aged 60-69 years. Similar increases in the incidence of Type 2 DM were also found in women. The most current epidemiological statistics of DM in Manitoba, or The Diabetes Burden of Illness Study (1995), indicated increasing

prevalence rates in aging populations of men and women with 1.4% for 25-39 year old category and 19.6% in the 70+-year-old category.

Prevalence of Type 2 DM is higher in Manitoba's aboriginal population. According to the Diabetes Burden of Illness Study (1995), almost 20% and 12% of Treaty-status Indian women and men, respectively had diabetes in 1991.

INSULIN BIOSYNTHESIS AND SECRETION

Biosynthesis

Insulin is synthesized in the pancreatic β-cell. The insulin precursor, preproinsulin, is synthesized in the ribosomal endoplasmic reticulum. During chain elongation the signal sequence is cleaved, thus resulting in proinsulin. Proinsulin, which is 86 amino acids long, is then transferred to the Golgi apparatus where it is packaged into immature secretory vesicles or storage granules (Nauck 1998). The granules are made acidic by proton pumps, which pump hydrogen ions into these granules. The acidic environment, in combination with an increased calcium concentration, activates two trypsin-like enzymes, called prohormone convertases. These enzymes cleave proinsulin into insulin, C-peptide and conversion intermediates (Nauck 1998).

The insulin protein, which has 51 amino acids, consists of two peptides, or one alpha and one β- chain, joined by two disulfide bonds (Chausmer 1998). Insulin is stored in the secretory vesicle until its secretion. In normal β-cells, mature secretory granules contain an insulin crystalline core and a mantle, consisting of C-peptide, minor secretory products and uncleaved proinsulin (Nauck 1998).

In normal β-cells, proinsulin is converted very efficiently to insulin and C-peptide, so upon secretion, a very small (5%) amount of proinsulin and intermediate products are secreted (Nauck 1998). However, it has been documented that the amount of proinsulin may be higher and represent a greater portion of insulin bioactivity in people with Type 2 DM and others who have insulin resistance as a result of another disease (Nauck 1998).

Glucose is the major stimulus for insulin biosynthesis, although the glucose concentration required for synthesis is much lower than is required for insulin secretion. Therefore, the rate of formation of insulin granules is not similar to the rate of insulin secretion (Nauck 1998).

Secretion

Mature storage vesicles may collect in the pancreatic β-cell cytoplasm or attach to the microtubular system of the β-cell (Roth and Kirchgessner 1981). Once stimulated, the vesicles move toward and fuse with the β-cell plasma membrane and release insulin into the extracellular space (Roth and Kirchgessner 1981).

Glucose and amino acids are the most potent stimulants for insulin secretion. Other participants in the regulation of insulin release are the cholinergic nervous system, gastric inhibitory protein (GIP), glucagon-like peptide-1 (GLP-1), glucagon, and arachidonic acid and its metabolites, such as leukotrienes and prostaglandins (Nauck 1998).

Glucose can stimulate insulin secretion either directly after uptake by the β-cell, or indirectly through the cholinergic nervous system that monitors plasma glucose levels.

In the β-cell, glucose is efficiently transported across the plasma membrane by a protein carrier, called Glut 2, and is immediately phosphorylated to glucose-6-phosphate by glucokinase. Glucose-6-phosphate can either go through glycolysis to generate energy for the β-cell or through the glucose-6-phosphate shunt (Nauck 1998). Glucose molecules that are diverted to the glucose-6-phosphate shunt increase the ATP/ADP ratio, which closes the ATP-dependent potassium channels that are present in the β-cell plasma membrane. Closure of these channels prevents the removal of potassium ions from the cell; therefore, the increased potassium ion concentration causes plasma membrane depolarization and the opening of voltage-dependent calcium channels (Nauck 1998). As a result, extracellular calcium flows into the β-cell cytoplasm, thus increasing the free intracellular calcium concentration. With increased cytoplasmic calcium concentration, the secretory vesicles translocate to the plasma membrane and eventually release insulin into the extracellular space (Nauck 1998).

Another product of glucose-6-phosphate shunt activity is NADPH. Increased NADPH concentrations are associated with greater availability of reduced glutathione as glutathione reductase permits NADPH to be recycled through its oxidation to NADP and oxidized glutathione to be recycled to its reduced form. Increased levels of reduced glutathione may cause the opening of L-type calcium channels to further increase the intracellular concentration of free calcium (Nauck 1998).

Amino acids can cause insulin secretion, since they can also be metabolized to make ATP and influence the ATP/ADP ratio in the β-cell. As well, some amino acids, such as arginine, are highly charged cations and may directly cause plasma membrane

depolarization and insulin secretion. Amino acids with the greatest insulin-stimulatory effects are L-arginine, L-leucine, and L-phenylalanine (Nauck 1998).

Plasma glucose concentrations will activate the cholinergic nervous system, resulting in direct activation of phospholipase C in the β-cell. Activated phospholipase C gives rise to inositol-1,4,5-triphosphate and diacylglycerol. Inositol-1,4,5-triphosphate mobilizes calcium from membrane-bound stores which also raises the free cytoplasmic calcium concentration. Diacylglycerol activates protein kinase C, which translocates to the β-cell plasma membrane. Diacylglycerol may also increase the membrane potential of the secretory granules so they can fuse with the plasma membrane and excrete insulin and other secretory products (Nauck 1998).

In the presence of glucose, glucagon, GIP and GLP-1 can augment insulin secretion through increasing adenosine 3′5′ monophosphate (cAMP) concentrations. Glucagon, GIP and GLP-1 bind to receptors on the β-cell plasma membrane and activate adenylate cyclase. High cAMP levels activate protein kinase A, which, in combination with protein kinase C, phosphorylate proteins to permit insulin exocytosis from the secretory granules. Arachidonic acid and its metabolites, such as leukotrienes and prostaglandins, may play a role in mediating the cAMP levels in the β-cell and thus influencing insulin secretion (Nauck 1998).

Role of Zinc in the Biosynthesis of Insulin

Scott (1934) discovered an association of zinc with crystalline insulin in the pancreas. Since this time, research has established zinc's role in insulin biosynthesis. Zinc appears to be involved once proinsulin is formed. Proinsulin is formed in the

endoplasmic reticulum after the cleavage of the signal peptide from preproinsulin. Proinsulin quickly folds into its three-dimensional structure and is transported to the Golgi apparatus. Both the transport medium and the Golgi apparatus are aqueous environments that contain zinc and calcium (Dodson and Steiner 1998). During transport to the Golgi apparatus, proinsulin molecules assemble into soluble hexamers containing two to four centrally-coordinated zinc ions (Emdin et al. 1980). Cleavage of the C-peptide causes the hexamer to be insoluble and results in the characteristic insulin granule formation in most animals (Dodson and Steiner 1998). Precipitation and crystalline formation of zinc insulin hexamers help favor the formation of insulin from proinsulin (Dodson and Steiner 1998). The zinc insulin hexamers remain in crystalline form until insulin secretion.

ZINC, INSULIN AND TYPE 2 DIABETES

Zinc and Insulin in Non-diabetic Animal Models

Zinc is an essential trace mineral that has many functions in the body. Zinc is a constituent of many metalloenzymes as a cofactor, or a stabilizer of the protein structure (Groff et al. 1995). Through these enzymes, zinc is involved in many metabolic processes involving carbohydrate, lipids and protein (Prasad 1983). Zinc is required in enzymes involved in DNA and RNA synthesis and their regulation. Alone, or through its association with metallothionein, and as a component of copper-zinc dismutase enzyme, zinc is important for cellular antioxidant defense (Prasad 1983, Groff et al. 1995, and Apostolova et al. 1997). Structural integrity, function and activities of some enzymes of cellular plasma membranes require zinc. Zinc stabilizes membrane phospholipids and

thiol groups and protects the membrane from lipid peroxidation (Bettger and O'Dell 1981, and Groff et al. 1995). Pancreas zinc and insulin, serum insulin and glucose, and glucose tolerance testing in zinc deficient animals will be discussed in the following sections.

Zinc has important functions in the pancreas. Acinar cells, in the exocrine pancreas, synthesize several digestive enzymes, including the zinc metalloenzyme, carboxypeptidase A, and store high concentrations of zinc (Case 1998). Alpha and β-cells of the endocrine pancreas also store zinc but not to the same extent. Dietary zinc manipulations have shown rapid changes in pancreatic zinc, thus indicating the pancreas a sensitive indicator of zinc status (Roth and Kirchgessner 1981). For example, decreased pancreatic zinc concentrations have been reported in normal glycemic animals fed zinc-deficient diets (Williams and Mills 1970, Roth and Kirchgessner 1981, Park et al. 1986, and Southon et al. 1988).

Pancreatic β-cell function, the focus of this study, is also influenced by zinc status. Zinc is important for pancreatic β-cell storage of insulin through the formation of zinc-insulin granules in storage vesicles (Roth and Kirchgessner 1981). Lowered pancreatic β-cell granulation was observed in Chinese hamsters fed a zinc-deficient diet (Boquist and Lernmark 1969). However, there is limited, conflicting evidence on the effect of zinc status on pancreatic insulin concentrations. Huber and Gershoff (1973) found no significant difference in pancreatic insulin concentrations of rats assigned to different dietary zinc treatment groups (1, 20, and 1200 ppm zinc). Brown and colleagues (1975) found significantly lower pancreatic insulin concentrations in rats fed a control diet compared to rats fed a zinc deficient diet or the pair-fed group. No

significant difference in pancreatic insulin concentration was found between zinc deficient and zinc control (pair-fed) groups.

Robinson and Hurley (1981) found a 25 –30% decrease in pancreatic insulin concentrations in fetuses of rats fed a zinc deficient diet ad libitum (0.4 ppm zinc) or a zinc supplemented diet (100 ppm zinc) in restricted amounts compared to fetuses of rats fed a zinc supplemented (100 ppm zinc) diet ad libitum. The researchers also found significantly lower β-cell numbers and a smaller proportion of β-cell occupancy in pancreata of fetuses from rats fed the zinc deficient diet compared to fetuses of both zinc supplemented groups. Based on these results, Robinson and Hurley (1981) concluded that zinc deficiency depresses β-cell proliferation more than other pancreatic cell types. Caloric restriction of the zinc supplemented group still permitted normal β-cell size and number; however, insulin concentration for each β-cell decreased, resulting in lowering pancreatic insulin concentration for this group.

Conflicting evidence on the effects of zinc deficiency on insulin secretion occur in both in vivo and in vitro studies. Boquist and Lernmark (1969) found elevated fasting serum immunoreactive insulin in zinc deficient Chinese hamsters compared to the zinc adequate control group. However, Huber and Gershoff (1973) found significantly lower fasting serum immunoreactive insulin in both zinc deficient and pair-fed control rats, when measured by an in vitro adipose tissue assay. Droke and associates (1993) did not find differences in fasting insulin concentrations in lambs fed different dietary zinc levels.

Droke and associates (1993) observed lower post-meal serum insulin concentrations after feeding lambs a zinc-deficient (3.7 ppm zinc) diet compared to lambs

fed a marginally zinc-deficient (8.7 ppm zinc) or zinc-adequate (43.7 ppm zinc) diet. No significant difference in insulin concentration was found between the marginally zinc-deficient and zinc-adequate groups. However, when subjected to an intravenous glucose tolerance test (0.15 g glucose/kg body weight), serum insulin concentrations of the zinc-deficient lambs were not significantly different from lambs that were fed the marginally zinc-deficient or the zinc-adequate diets (Droke et al. 1993).

Quarterman and colleagues (1966) found significantly lower plasma insulin concentrations in zinc deficient rats compared to zinc control (pair-fed) rats when subjected to an intraperitoneal glucose (700 mg/ 100 g body weight) tolerance test. However, other studies found no difference in serum insulin concentrations of zinc deficient animals fed ad libitum compared to pair-fed zinc adequate animals and animals in other dietary zinc treatment groups (Boquist and Lernmark 1969, Brown et al. 1975, and Quarterman and Florence 1972). Through an intraperitoneal glucose tolerance test, Park and associates (1986) found higher serum insulin concentrations in rats force-fed a zinc deficient diet compared to rats fed a zinc replete (control) diet or rats fed the zinc-deficient diet ad libitum.

Zinc deficiency reduces glucose tolerance or the ability to handle a glucose load as would be found in a meal. Fasting glucose levels were not significantly different in zinc-deficient rats compared to rats on zinc-adequate diets (Quarterman et al. 1966, Quarterman and Florence 1972). However, elevated fasting plasma glucose levels were found in rats force fed a zinc deficient diet by gastric intubation (Park et al. 1986).

Zinc-deficient animals had impaired glucose tolerance when subjected to intraperitoneal (Boquist and Lernmark 1969) and intravenous glucose tolerance tests

(Boquist and Lernmark 1969, and Huber and Gershoff 1973). Boquist and Lernmark (1969) demonstrated that plasma glucose concentrations of zinc-deficient Chinese hamsters quickly became higher and almost double the glucose concentrations of the zinc-adequate hamsters thirty minutes after an intraperitoneal glucose injection (2 g/kg body weight). Glucose concentrations of the zinc-deficient Chinese hamsters did not return to basal values until close to eight hours after the glucose injection. These results occurred after the Chinese hamsters were fed a zinc-deficient diet for one week. Boquist and Lernmark (1969) also found similar responses in zinc-deficient Chinese hamsters compared to zinc-adequate Chinese hamsters thirty minutes after an intravenous injection of glucose.

Quarterman and colleagues (1972) found no significant difference in glucose tolerance between zinc-deficient and control rats when subjected to an intraperitoneal glucose tolerance test. However, the glucose dose (0.175 g/ 100 g body weight) used for this test has been criticized for being too low to cause sufficient glucose intolerance.

There are mixed results on the glucose response of zinc-deficient animals subjected to an oral glucose tolerance test. Hendricks and Mahoney (1972) found no significant differences in oral glucose tolerance between zinc-deficient and zinc-adequate rats fed 0.250 g glucose / 100 g body weight by gastric intubation. Brown and associates (1975) similarly found no significant difference between zinc-deficient and control rats in glucose tolerance when fed 0.500 g glucose/ 100 g body weight by gastric intubation. There have been suggestions that differences in responses observed in oral glucose tolerance tests, and intravenous and intraperitoneal glucose tolerance tests may be due to greater oral stimulation of insulin secretion (Roth and Kirchgessner 1981).

Park and colleagues (1986) observed that zinc-deficient force fed rats had significantly impaired glucose tolerance thirty minutes after an oral glucose load when compared to rats fed zinc replete, force fed (control) diet or fed the zinc deficient diet ad libitum. With the exception of a zinc-deficient group fed ad libitum, both zinc-deficient force-fed and zinc replete force-fed rats were fed by gastric intubation. Significantly higher glucose intolerance was still present in the zinc-deficient force fed animals ninety minutes after the administration of the glucose load. However, no significant difference in glucose response was observed in the oral test between zinc-replete force-fed rats and zinc-deficient rats fed ad libitum. These researchers suggested that rats on the zinc-deficient diet ad libitum developed the cyclic eating patterns and decreased food intake, which is characteristic in zinc-deficient animals, as a protective mechanism to slow down the progression of zinc deficiency. Rats force fed the zinc-deficient diet did not have the opportunity to adapt to use this protective mechanism that resulted in the quick deterioration of their zinc status (Park et al. 1986).

Another interesting result found in the Park and colleagues (1986) study evolved from the histological examination of rat pancreata from each diet group. The researchers found no difference among all groups; pancreata from zinc-deficient force fed zinc deficient ad libitum and zinc-replete force fed animals had normal histology. This led the researchers to speculate that the impaired glucose tolerance observed in the zinc-deficient force fed rats was not due to decreased synthesis or secretion of insulin, but rather caused by peripheral resistance to insulin. Zinc-deficient, force fed rats may have altered membrane structure and function causing impaired glucose utilization (Park et al. 1986). Two limitations of this study were the researchers did not measure pancreatic insulin concentrations or insulin sensitivity.

Although the focus of this thesis will be the interaction of zinc and insulin in pancreatic β-cell function, it is acknowledged that there are interactions of zinc and insulin in the periphery. Arquilla and associates (1978) found that zinc is important in stabilizing the insulin conformation for insulin receptor recognition and binding to insulin. The stability provided by zinc also protected insulin from enzymatic degradation by liver plasma membranes (Arquilla et al. 1978).

Zinc Status and Type 2 Diabetes

Altered zinc metabolism and tissue zinc status in diabetes mellitus has been documented in some, but not all, animal and human studies. Excessive urinary zinc excretion (hyperzincuria) is one parameter that is associated with DM. All studies agree that higher zinc excretion occurs in diabetic animals compared to their nondiabetic controls (Failla and Kiser 1981, Levine et al. 1983, and Simon 1998). Similarly, higher urinary zinc excretion has been found in humans with DM compared to non-diabetic humans (Pidduck et al. 1970, Kinlaw et al. 1983, Sjorgren et al. 1988, Walter et al. 1991, and Taneja et al. 1998). Increased levels of zinc excretion were found in first-degree relatives of people with DM (Pidduck et al. 1970). Taneja and colleagues (1998) found a significant increase in urinary zinc excretion in daughters of DM parents compared to daughters of non-DM parents.

With the exception of Simon (1998) commenting that hyperzincuria did not seem to be influenced by dietary zinc intake, other animal studies have not reported potential associations between urinary zinc excretion and other diabetic parameters. In human studies, correlation tests between urinary zinc excretion and other measures have yielded

variable results. Kinlaw and associates (1983) found hyperzincuria to be positively correlated with serum glucose and proteinuria but not correlated with glycosylated hemoglobin. However, Walter and colleagues (1991) found no consistent correlation between hyperzincuria and plasma glucose or proteinuria. Walter and colleagues (1991) and Sjorgren and associates (1988) also found no correlation between hyperzincuria and glycosylated hemoglobin.

Disagreements also occur in determining the effect of DM on plasma or serum zinc status. Observations in animal studies vary from significantly higher (Failla and Kiser 1981), no significant difference (Simon 1998), and significantly lower (Levine et al. 1983, and Donaldson et al. 1988) plasma or serum zinc in diabetic animals compared to their controls. Possible reasons for this discrepancy could be genetic effects where different animal models for DM may be affected differently by the disease. Failla and Kiser (1981) chemically induced Type 1 diabetes in Sprague-Dawley rats with streptozotocin (STZ, induces pancreatic β-cell destruction) that could cause different responses to diabetes than animals that are genetically prone to developing DM. Other reasons for plasma or serum zinc discrepancies in the animal studies could be age of the animals, length of study, and concentration of zinc in the diet.

Human studies also show discrepancies in plasma or serum zinc values of DM compared to non-DM subjects. Some studies found decreased plasma or serum zinc concentrations (Sjogren et al. 1988, Winterberg et al. 1989, Walter et al. 1991, and Blostein-Fujii et al. 1997). Raz and colleagues (1988) found higher serum zinc levels in DM subjects compared to their non-diabetic controls. Other studies found no significant differences in plasma or serum zinc concentrations (Pidduck et al. 1970, Niewoehner et

al. 1986, Kinlaw et al. 1983, and Ruiz et al. 1998). Niewoehner and associates (1986) found a small percentage (9%) of DM volunteers had low serum zinc levels. Although there are mixed conclusions about the effect of DM on plasma or serum zinc, it appears that some people with DM may be more susceptible to a decreased zinc status in this tissue.

No consistent correlations of plasma or serum zinc concentration to other measured parameters were found in human studies comparing DM and non-DM volunteers. Winterberg and colleagues (1989) found serum zinc concentration was negatively correlated to glycosylated hemoglobin levels, and positively correlated with duration and complications of the disease. Other studies did not find significant correlation of serum zinc (Kinlaw et al. 1983 and Niewoehner et al. 1986) or plasma zinc (Sjogren et al. 1988 and Ruiz et al. 1998) with glycosylated hemoglobin levels.

Animal studies indicate a redistribution of zinc in other tissues of animals that are chemically induced or are genetically prone for diabetes. Again, controversy surrounds the zinc status of certain tissues; however, the studies do indicate that DM influences tissue zinc status. Failla and Kiser (1981) found greater zinc concentrations in livers and kidneys of diabetic Sprague-Dawley rats induced by STZ compared to their non-diabetic controls. Failla and Gardell (1985) again demonstrated greater liver and kidney zinc concentrations in diabetic-prone BB Wistar rats compared to their non-diabetic controls. Donaldson and colleagues (1988) found similar results in db/db mice. Simon (1998) found greater kidney zinc concentrations in db/db control mice when compared to their lean controls. Bégin-Heick and associates (1985) found greater liver zinc concentrations

in ob/ob mice compared to their controls. However, Levine and colleagues (1983) found no significant difference in db/db mice.

Animal studies also indicate a change in femur zinc status. The femur is considered to be a long-term indicator of zinc status (Roth and Kirchgessner 1981). Significantly lower femur zinc concentrations were found in diabetic animals when they were compared to their non-diabetic controls (Levine et al. 1983, Bégin-Heick et al. 1985, Donaldson et al. 1988, Southon et al. 1988, and Kennedy and Failla 1987). However, other studies found no significant difference in femur zinc concentrations (Failla and Gardell 1985, and Simon 1998). Observed differences in femur zinc concentrations could be due to age of the animals, length of the study and concentration of zinc in the diet.

Not much research has investigated pancreatic zinc status in either human or animal diabetic studies. Scott and Fisher (1938) found that pancreata of diabetic cadavers had fifty percent less zinc than pancreata of non-diabetic cadavers. Conflicting results are found in animal studies. Failla and Gardell (1985) found that zinc concentration was 20% higher in the pancreata of diabetic BB Wistar rats compared to their controls. However, Bégin-Heick and associates (1985), Southon and colleagues (1988), and Simon (1998) found decreased pancreatic zinc concentrations in their diabetic animals compared to controls.

Zinc Supplementation in Type 2 Diabetes

Several human and a few animal studies have looked at the effect of zinc supplementation on tissue zinc status in DM, as well as the initiation and complications of the disease. All human studies observed increased plasma or serum zinc status after

zinc supplementation (Niewoehner et al. 1986, Blostein-Fujii et al. 1997, Raz et al. 1989, and Winterberg et al. 1989). However, opinions vary on whether zinc supplementation will benefit humans with DM. Although Blostein-Fujii and colleagues (1997) found increased plasma zinc in type 2 DM post-menopausal women after zinc supplementation (30 mg zinc/day as amino acid chelate for 3 weeks), the researchers did not observe a significant increase in plasma 5′ nucleotidase activity, a zinc metalloenzyme. Winterberg and associates (1989) found significantly reduced fasting serum glucose concentrations in adult volunteers with type 1 DM on oral zinc supplementation (50 mg zinc oxide/day for 3 weeks) compared to no zinc supplementation. Raz and colleagues (1988) found that insulin secretion decreased in an intravenous glucose tolerance test and that average glucose disposal worsened in volunteers with type 2 DM who took 220 mg zinc sulfate/day for seven to eight weeks. The researchers assumed no significant changes occurred in pancreatic β-cells as a consequence of zinc supplementation and concluded zinc treatment could aggravate the DM condition (Raz et al. 1989).

Niewoehner and associates (1986) observed volunteers with type 2 DM who were taking oral zinc supplements (220 mg zinc sulfate/day for six to eight weeks) had increased lymphocyte response to phytohemagglutinin, a mitogen. These findings indicate a possible role of zinc supplementation in improving immune function. However, Raz and colleagues (1989) found no significant difference in lymphocyte response in zinc supplemented type 1 DM subjects compared to control volunteers. Raz and colleagues (1989), Winterberg and associates (1989) and Niewoehner and colleagues (1986) all found the level of glycosylated hemoglobin unchanged after zinc supplementation.

Few animal studies have investigated the effect of zinc supplementation on tissue zinc status and diabetic indices in animal models for DM. Simon (1998) found higher femur zinc concentrations in zinc supplemented (300 ppm) db/db mice versus db/db mice fed a zinc control diet. This finding is in contrast to the Bégin-Heick and colleagues (1985) study that zinc supplementation (1000 ppm) did not significantly affect femur zinc in ob/ob mice. Simon (1998) explained the discrepancy could be the result of the different animal models and ages of the animals in the two studies. Simon (1998) further hypothesized that using weanling mice in her study may have permitted recovery of depleted bone mineral, such as zinc. Using adult mice, such as those used in the Bégin-Heick et al. (1985) study may have made bone mineral recovery impossible (Simon 1998).

Zinc supplementation elevates pancreatic zinc concentrations in diabetic animal models (Simon 1998, Tobia et al. 1998). Bégin-Heick and colleagues (1985) and Tobia and associates (1998) found higher pancreatic insulin concentrations in diabetic animals fed a zinc supplemented diet compared to their zinc adequate controls. It appears that zinc supplementation may improve pancreatic insulin biosynthesis and storage in diabetic animals.

Both in vivo and in vitro studies demonstrated that zinc supplementation raises pancreatic metallothionein (MT) concentrations (Andrews et al. 1990, Yang and Cherian 1994, Ohly and Gleichmann 1995, and Apostolova et al. 1997). Metallothionein is a cytosolic, low molecular weight protein that is found in many tissues of the body, including the red blood cell, intestine, liver, kidney and pancreas (Groff et al. 1995 and Ohly and Gleichmann 1995). The high content of cysteine residues (30% residues)

enables MT to have a high binding capacity for metals such as cadmium, zinc and copper (Groff et al. 1995, and Apostolova et al. 1997). Because MT has a strong affinity for these metals, researchers believe MT plays a role in detoxifying these metals and there are speculations that MT "...regulates [zinc] and [copper] homeostasis..." (Apostolova et al. 1997).

Andrews and colleagues (1990) found that both endocrine and exocrine portions of pancreata from Sprague Dawley rats had higher MT mRNA levels, four hours after one subcutaneous zinc injection (200 μmol/ kg body weight). Acinar cells had more intense MT immunostaining, 24 hours after the injection, suggesting an accumulation of MT in acinar cells. However, metallothionein immunostaining was not visible in pancreatic islets (Andrews et al. 1990). An in vitro study conducted by Ohly and Gleichmann (1995) showed that zinc induction of MT in the pancreas was dose-dependent.

Studies have suggested a possible role of MT as a scavenger of oxygen free radicals, thus MT may be an important cellular defense mechanism against free radicals (Yang and Cherian 1994, and Ohly and Gleichmann 1995). This theory is being tested in studies that induce beta-cell destruction and diabetes with STZ, a compound that helps cause oxygen free radical generation (Ohly and Gleichmann 1995). Studies that investigated the protective effects of MT on STZ-diabetes-induced rats found that rats pretreated with zinc 4 and 24 hours before the STZ injection had higher MT levels and had less severe diabetes (Yang and Cherian 1994, and Apostolova et al. 1997).

Apostolova and associates (1997) also found that zinc alone protected pancreatic beta-cell function in MT-null mice from the effects of STZ, even at the lowest injection

dose (1 mg zinc/ kg body weight). Pretreatment of MT-null mice with 1 mg zinc/ kg body weight prior to the STZ injection resulted in lower plasma glucose levels and similar insulin and glucagon levels compared to MT-null mice that received STZ injection only. Zinc pretreatment of 10 mg/ kg body weight in C57BL/6J (control) mice before STZ injections yielded similar results. Apostolova and associates (1997) concluded that zinc alone can also provide protection against STZ-induced diabetes.

Bégin-Heick and colleagues (1985) found significantly lower fasting plasma glucose and plasma insulin concentrations in genetically obese (ob/ob) mice fed a zinc supplemented diet (1000 ppm for four weeks) compared to ob/ob controls. However the fasting plasma glucose and insulin levels in zinc supplemented ob/ob mice were higher than the lean controls. Tobia and associates (1998) found similar results when non-fasted plasma glucose concentrations from zinc supplemented (1000 ppm) and control diabetic BB Wistar rats were compared. Bégin-Heick and colleagues (1985) observed that zinc supplementation did not significantly change plasma glucose concentrations and insulin secretory response to a glucose load in ob/ob mice during the glucose tolerance test. This observation led these researchers to conclude that zinc supplementation did not improve peripheral glucose tolerance in ob/ob mice. However, Tobia and associates (1998) found that zinc supplemented BB Wistar rats had both significantly lower plasma glucose concentrations and lower insulin secretory release during the glucose tolerance test. Both studies used glucose loads of 1 g/ kg body weight for intraperitoneal glucose tolerance tests in animals of similar age (100 days old). Animals (ob/ob mice) studied by Bégin-Heick and colleagues (1985) are used to study type 2 DM, whereas diabetes-prone BB Wistar rats used by Tobia et al. (1998) are used to study type 1 DM, which could explain

the discrepancies in these results.

Zinc supplementation appears to delay the onset of DM in diabetes-prone animals. Tobia and associates (1998) found the onset of diabetes occurred at a much older age in zinc supplemented animals compared to zinc adequate controls. However, once the zinc-supplemented animals became diabetic, there was no significant difference in the severity of the disease compared to BB rats on the control diet. This was based on a similar level of severe islet cell inflammation and decreased insulin staining in all diabetic animals, regardless of diet. These results, in addition to the observation of decreased pancreatic insulin release, led Tobia and associates (1998) to conclude that zinc supplementation protects the pancreas by attenuating insulin secretion and decreasing free radical injury of pancreatic β-cells. This action prevents beta-cell exhaustion and preserves beta-cell structure and function (Tobia et al. 1998). However, once an animal becomes diabetic, the progression of the disease is not delayed by zinc supplementation.

THE n-3 FATTY ACIDS AND TYPE 2 DIABETES

Obesity is one of the most important risk factors for insulin resistance and for people genetically predisposed for type 2 DM. Immoderate dietary fat intake is the primary nutritional risk factor for obesity (Vessby 1995). Compared to people with normal glycemic control, higher dietary fat intake has been reported in people with type 2 DM and impaired glucose tolerance (Vessby 1995).

Dietary Lipid, n-3 Fatty Acids and Non-diabetic Animals

The association of higher fat intakes and insulin resistance has been investigated in rodents. Short-term and long-term high fat (59-72% of total calories) studies using non-diabetic animal models have shown higher fat accumulation with reduced insulin sensitivity in both skeletal muscle and adipose tissue (Lemonnier et al. 1974, Stern et al. 1975, Grundleger and Thenen 1982, and Storlein et al. 1986). Storlein and colleagues (1986) found that feeding high fat diets to Wistar rats suppressed glucose uptake in skeletal muscle and brown adipose tissue with some resistance to the suppression of hepatic glucose output. Grundleger and Thenen (1982) observed impaired glucose transport in soleus muscle of lean Zucker rats fed a high fat diet for 10 days. Other observations include higher muscle triglyceride storage, decreased insulin receptor binding to insulin with impairment of tyrosine kinase activation in animals fed high fat diets (Storlein 1996).

In addition to total fat intake, fat type has also been found to be important in influencing peripheral insulin sensitivity. Higher intakes of saturated fatty acids appear to directly cause peripheral insulin resistance, or indirectly through increased adiposity (Storlein et al. 1996). Beneficial effects on peripheral insulin sensitivity and insulin action have been found in rat studies where n-3 fatty acids, mostly from fish oil, were included in the high fat diets (Storlein et al. 1987, Storlein et al. 1991 and Luo et al. 1996). Incorporation of n-3 fatty acids into plasma membrane phospholipids in skeletal muscle has been speculated to be important for efficient insulin action (Storlein et al. 1990). Luo and associates (1996) found insulin action was positively correlated with the fatty acid unsaturation index in membrane phospholipids.

The n-3 fatty acids have hypolipidemic effects by lowering serum very low density lipoprotein (VLDL), triglyceride, and lipoprotein a concentrations (Simopoulos 1999). Topping and colleagues (1987) reported that n-3 fatty acids inhibited hepatic overproduction of VLDL-triglycerides, thus attenuating hypertriglyceridemia in non-diabetic rats fed standard rat chow supplemented with 8% (w/w) fish oil compared to rats fed rat chow supplemented with 8% safflower oil.

n-3 Fatty Acid Metabolism

The n-3 fatty acids, a focus of this thesis, are one of two groups of essential fatty acids (EFA) that must be obtained from the diet. Very long chain (20 or more carbons in length) n-3 fatty acids such as eicosapentaenoic acid (EPA) (C20:4 n-3) and docosahexaenoic acid (DHA) (C22:6 n-3) are obtained from fatty fish (salmon, mackerel, herring, tuna) and shellfish (Groff et al. 1995, and Haumann, 1997). Alpha-linolenic acid (ALA) is a long chain (18-20 carbons in length) n-3 fatty acid and a precursor to EPA and DHA. ALA is found in food sources such as leafy vegetables and vegetable oils, such as soy, canola, rapeseed and flaxseed (Groff et al. 1995). Flaxseed oil contains approximately 55-57% ALA (Carter 1993).

Each n-3 fatty acid has "the first double bond located at the third carbon from the methyl end of the hydrocarbon chain" (Haumann 1997). Desaturation and elongation of ALA forms EPA and DHA, and further reactions catalyzed by cycloxygenase and lipoxygenase form the 3-series eicosanoids such as prostacyclins, thromboxanes and leukotrienes (Groff et al. 1995) (*Figure 1*). ALA competes with linoleic acid (LA), an n-6 fatty acid, for the same enzymes for biosynthesis of long chain fatty acids and

eicosanoids. However, most ALA and dietary EPA and DHA are preferentially oxidized for fuel by mitochondria and peroxisomes, respectively, through β-oxidation (Opara and Hubbard 1993). Newly synthesized and remaining dietary EPA and DHA are incorporated into membrane phospholipids similarly to AA and long chain n-6 fatty acids (Opara and Hubbard 1993).

n-3 Fatty Acids and Type 2 Diabetes

Research has focused on the effects of n-3 fatty acids on the various characteristics of type 2 DM, which include hypertriglyceridemia, insulin resistance, and hyperglycemia (Simopoulos 1999). Similar hypolipidemic effects of n-3 fatty acids have been observed in both diabetic animal and human studies. Luo and colleagues (1996) observed significantly lower plasma triglycerides, plasma cholesterol and plasma phospholipids in Sprague-Dawley rats fed a fish oil diet compared to rats fed a control diet consisting of vegetables and oils. The Sprague-Dawley rats became diabetic (fasting blood glucose around 7.70 to 7.76 mmol/L) when fed diets consisting of 50% (calories) sucrose and 30% (calories) fat for six weeks. The researchers also observed significantly lower fasting plasma insulin concentrations in the fish oil group.

The C57BL/6J mouse is a Type 2 DM model when fed a high fat diet. Ikemoto and associates (1996) found significantly lower fasting plasma triglyceride concentrations in C57BL/6J mice fed high fat (60.2% calories) diets containing either perilla oil or fish oil compared to mice on a high carbohydrate diet. Perilla oil has approximately 62% ALA (Ikemoto et al. 1996). Plasma cholesterol did not significantly change in this study.

Significantly lower plasma triglyceride concentrations are commonly observed in

humans with type 2 DM who consume n-3 fatty acids (Popp-Snijders et al. 1987, Rivellese et al. 1996, and Luo et al. 1998). Rivellese and associates (1996) also observed significant reduction in plasma VLDL triglyceride, and nonesterified fatty acid concentrations in type 2 DM subjects who took fish oil supplements for six months compared to type 2 DM subjects who consumed the placebo. During the six month trial, diabetic subjects took fish oil supplements containing 2.7 grams of EPA and DHA for two months and then took supplements containing 1.7 grams of EPA and DHA for four months.

There are conflicting results from studies investigating the effect of n-3 fatty acid supplementation on glycemic control in people with type 2 DM. Vessby and colleagues (1992) found significantly higher fasting and post-prandial blood glucose concentrations in type 2 DM subjects consuming diets containing 4 to 5 grams of long chain n-3 fatty acids, derived from polyunsaturated fatty acids from fish, and 1.5 grams of short chain n-3 fatty acids, derived from ALA, for 3 weeks. Fat intake of these individuals was 31% of total calories. Since serum concentrations of triglycerides, cholesterol, VLDL-triglycerides, and VLDL-cholesterol were significantly lower in this group, Vessby and associates (1992) concluded that long chain n-3 fatty acids consumed as part of a moderate fat diet have both beneficial and adverse effects in type 2 DM.

It is possible that the amount of n-3 fatty acids (6 grams) in the Vessby (1992) study were excessive, which might have caused adverse effect on blood glucose concentrations. However, Luo and colleagues (1998) did not find deleterious effects of long chain n-3 fatty acids from fish oil on glycemic control. In this study, type 2 DM subjects took 6 grams of fish oil. Rivellese and associates (1996) found lower amounts of

long chain n-3 fatty acids in the form of EPA and DHA (2.7 grams for two months and 1.7 grams for 4 months) did not produce deterioration of blood glucose control in Type 2 DM subjects.

Dietary Lipid, n-3 Fatty Acids and the Pancreas

As stated earlier, fatty acids are involved in glucose stimulated insulin secretion (GSIS) in the pancreas (Nauck 1998) (Please see *Insulin Biosynthesis and Secretion*). However, varied insulinotropic effects on GSIS are observed with different fatty acids (Opara and Hubbard 1993). Stein and colleagues (1997) found insulinotropic effects of fatty acids on GSIS were affected by degree of unsaturation and chain length. After testing five different fatty acids (octanoate (C8:0), linoleate (C18:2 n-6), palmitate (C16:0), oleate (C18:1 n-9), and stearate (C18:0)) in the perfusate, palmitate and stearate produced the highest GSIS of fasted rat pancreata perfused with 12.5 mM glucose (Stein et al. 1997). Octanoate and linoleate, short chain saturated fatty acid and polyunsaturated fatty acid, respectively, produced the lowest GSIS in this study. Similar results were observed in perfused fed rat pancreata at both basal and stimulatory glucose concentrations. These observations led Stein and colleagues (1997) to conclude that pancreatic insulin secretion is influenced by free fatty acid composition in addition to total free fatty acid in solution surrounding the pancreas. However this study is limited since insulinotropic effects of n-3 fatty acids were not tested.

Fatty acid stimulated insulin secretion of perfused rat pancreata was observed when a mixture of LA and ALA fatty acids (n-6: n-3, 2:1) was added to a solution containing a basal glucose concentration (Opara and Hubbard 1993). However, no

indication was given for the effect of this fatty acid mixture on the level of pancreatic insulin secretion at glucose concentrations required for GSIS.

In vitro animal studies have shown that circulating triglyceride and free fatty acid concentrations can influence pancreatic sensitivity and alter β-cell function. *In vitro* studies have shown impaired GSIS in rat pancreatic islets after long-term exposure to palmitate, a saturated free fatty acid (Zhou and Grill 1994). Loss of GSIS and lowered GLUT-2 glucose transporter activity were observed in pancreata from seven week old obese Zucker diabetic fa/fa (ZDF-drt) rats, with free access to standard rat chow, two weeks prior to developing hyperglycemia (Lee et al. 1994). During this time, plasma triglyceride concentration was significantly higher in ZDF rats than in obese fa/fa nondiabetic and lean controls. A significant correlation was found between islet cell triglyceride content and plasma free fatty acid concentrations (Lee et al. 1994).

Normal (lean Wistar) rat islets cultured in 2 mM solution of long chain fatty acids (oleate/palmitate, 2:1) for seven days had increased insulin secretion at basal glucose concentrations. However, the same islet cells, as well as prediabetic (ZDF- drt) islet cells had 68% and 69%, respectively, lower GSIS compared to controls not cultured in free fatty acid (Lee et al. 1994). Millburn and associates (1995) found significantly increased insulin secretion and glucose usage in lean Wistar islets cultured in 1 mM and 2 mM solutions of long chain free fatty acids (oleate/palmitate, 2:1). These results led Millburn and associates to conclude that high free fatty acid concentrations can induce insulin hypersecretion and β-cell hyperplasia in pancreatic islets of lean Wistar rats.

The fa/fa Zucker Rat as a Model of Obesity and the Prediabetic State

The fa/fa Zucker rat is a genetic model of obesity that is originally derived from a spontaneous mutation that occurred from cross-breeding Merck Stock M and Sherman rats (Bray 1977). The model is used to study obesity and has characteristics of the prediabetic state prior to Type 2 DM such as hyperinsulinemia, peripheral insulin resistance and high blood lipid concentrations. The genetic mutation is in an autosomal, recessive gene that exists on chromosome 5, "which shows homology with the mouse diabetic (db) gene" (Zucker and Zucker 1961 and Mathé 1995), that is, the leptin receptor. Homozygous, recessive fa/fa Zucker rats have hyperinsulinemia, hyperphagia, hyperlipidemia, and low glucagon secretion. Hyperlipidemia in these animals results from high serum concentrations in all lipoproteins for cholesterol and from high VLDL concentrations for triglyceride (Mathé 1995). Hyperphagia contributes to the obesity of fa/fa Zucker rats; however, strict dietary control will not completely prevent these animals from developing obesity (Zucker and Zucker 1961, and Mathé 1995). Heterozygotes for the fatty trait are lean, have normal blood lipid values, and cannot be distinguished phenotypically from homozygous Zucker lean rats (Bray 1977, and Mathé 1995).

In contrast to lean Zucker rats, the fa/fa Zucker rats also have hyperleptinemia similar to db/db mice (Caro et al. 1996). Leptin, a hormone synthesized by the ob gene in the adipose tissue, depresses appetite and thus adipose tissue accumulation (Caro et al. 1996, Friedman 1998, and reviewed by Tallman and Taylor 1999). Both animal and human studies have demonstrated that circulating leptin concentrations strongly correlate

with body fat (Caro et al. 1996 and Friedman 1998). Serum leptin concentrations are believed to be determined primarily by visceral fat tissue (reviewed by Tallman and Taylor 1999). Normally, as the amount of adipose tissue increases, more leptin will be synthesized as a signal to the brain to raise energy expenditure and lower food intake. The reverse occurs when the amount of adipose tissue is lowered (Caro et al. 1996, and Friedman 1998). Research has indicated that several areas in the brain may be a target of leptin; high affinity for binding leptin was demonstrated in the rat hypothalamic plasma membranes (Caro et al. 1996).

Hyperleptinemia can be caused by a mutation in the DNA coding (the ob gene) for the leptin protein, or a mutation in the DNA coding for the leptin receptor (Ob-R) (Caro et al. 1996, and Friedman 1998). Parabiosis experiments, along with insensitivity of db/db mice to leptin, indicated that the db gene encodes for Ob-R (Friedman 1998). Similar mutations in the fa/fa Zucker rats are found in the Ob-R, which are homologous to the db mutation (Friedman 1998).

There is evidence that many obese people have high blood leptin concentrations (reviewed by Tallman and Taylor 1999) and studies have suggested a possible association between human obesity and leptin insensitivity (Friedman 1998). One study found positive correlations between serum leptin concentrations and fasting insulin or proinsulin concentrations, and a negative correlation with the ratio between proinsulin and insulin (Haffner et al. 1998). Understanding hyperleptinemia in the fa/fa Zucker rat may also provide insight into human hyperleptinemia and obesity.

The characteristics of the fa/fa Zucker rat during its lifecycle are presented in Table 1. The fatty trait is identified when Zucker rats are two to four weeks old by

hyperphagia, and elevated plasma insulin, triglyceride and LDL levels (Zucker and Zucker 1961, Zucker and Antoniades 1972, York et al. 1972 Shino et al. 1973, and Bray 1977). Elevated blood lipids cause the characteristic lactescence (milky appearance) of the fa/fa Zucker serum (Zucker and Zucker 1961, and Shino et al. 1973). By five weeks of age, fa/fa Zucker rats have "visible and palpable differences in body fat content and in body shape" (Zucker and Zucker 1961, and Bray 1977). Hypertrophy and hyperplasia of fa/fa Zucker rat adipocytes start at a young age and continue throughout the lifespan (Bray 1977).

Plasma insulin concentrations of fa/fa Zucker rats are normal at weaning (two weeks of age), but increase to six-fold higher (400 μUnits/mL) than lean controls at 15 weeks old. Plasma insulin concentrations eventually drop to 200 μUnits/mL in older fa/fa Zucker rats (Zucker and Antoniades 1972). Adipocyte hyperplasia and hypertrophy correlates well with plasma insulin concentrations since maximum percentage of body lipid and fat cell size also occur when these rats are 15 weeks old (Zucker and Antoniades 1972). The pancreatic β-cells undergo hypertrophy and hyperplasia as early as eight weeks old in the fa/fa Zucker rat (Shino et al. 1973, Larsson et al. 1977, Mathé 1995, and Chan et al. 1998). Maximum β-cell hyperplasia with β-cell formation from extrainsular cells occurs around 20 weeks (Shino et al. 1973, and Larsson et al. 1977).

Normal basal glucose concentrations of fa/fa Zucker rats occur early and late in life; however, elevated basal glucose concentrations after a prolonged fast were observed in 20 week old fa/fa Zucker rats (Zucker and Antoniades 1972). Normal glucose tolerance curves are observed in both young and old fa/fa Zucker rats following an intravenous or intraperitoneal glucose administration (Zucker and Antoniades 1971, and

Ionescu et al. 1985). However, fa/fa Zucker rats have impaired glucose tolerance when subjected to an oral glucose tolerance test that appears to worsen with age (Ionescu et al. 1985). Impaired glucose tolerance with increasing age is also observed in lean Zucker rats. Glucose intolerance and elevated fasting blood glucose concentrations become normalized in fa/fa Zucker rats around age 26 to 50 weeks (Zucker and Antoniades 1972 and Stern et al. 1972).

To offset increasing peripheral insulin resistance and to maintain normal blood glucose concentrations, pancreatic β-cells of fa/fa Zucker rat undergo hypertrophy and hyperplasia when the rats are approximately six and eight weeks old, respectively (Shino et al. 1973, and Chan et al. 1998). Most of the β-cells are degranulated, indicating insulin hypersecretion from these cells (Shino et al. 1973). Pancreatic β-cells are extremely hypertrophied and have pronounced hyperplasia in fa/fa Zucker rats at 15 to 24 and 20 weeks of age, respectively (Shino et al. 1973, and Larsson et al. 1977). Isolated pancreatic islets from fa/fa Zucker rats are heterogeneous in size with large islets primarily responsible for insulin hypersecretion (Hayek and Woodside 1979, and Chan et al. 1998). Smaller islets of the fa/fa Zucker rat synthesize similar concentrations of insulin and have normal functioning that is comparable to the pancreatic islets of lean rats (Chan et al, 1998).

Fibrosis of the islets also occurs between 15 to 24 weeks with increased fibrosis evident when fa/fa Zucker rats are 52 weeks of age (Shino et al. 1973). As well, β-cells appear granulated, hyperinsulinemia disappears and β-cell hyperplasia decreases (Shino et al. 1973, and Larsson et al. 1977). Pancreatic β-cell function is greatly reduced. Dietary intervention appears to have a protective role against the development of

pancreatic islet cell lesions and preserve pancreatic β-cell function. Restoration of pancreatic islets to normal morphology was observed in diet-restricted fa/fa Zucker rats (Larsson et al. 1977).

The fa/fa Zucker rat develops focal glomerulosclerosis (FGS) in the kidneys at an early age, which ultimately leads to renal failure and death (Kasiske et al. 1987). Albuminuria is observed in fa/fa Zucker rats at about 12 weeks of age, but renal function is similar to the renal function of lean Zucker rats (Kasiske et al. 1987). Elevated albuminuria and the development of kidney lesions occur at 18 and 35 to 40 weeks, respectively (Zucker and Zucker 1961, Zucker and Zucker 1962, and Kasiske et al. 1987). Depending on the diet, fa/fa Zucker rats do not live past 18 months (Zucker and Zucker 1962, Larsson et al. 1977, and Kasiske et al. 1987).

Reproductive function is limited in fa/fa Zucker rats (Bray 1977). Female fa/fa Zucker rats are unable to be impregnated, since they have "...delayed vaginal opening, prolonged estrus cycles, and decreased uterine weight..." (Saiduddin et al. 1973). If placed on a restricted chow diet, male fa/fa Zucker rats can sire litters (Bray 1977). Therefore, heterozygous Zucker rats (Fa/?), or male fa/fa Zucker rats and female heterozygous Zucker rats are used in breeding programs (Bray 1977).

II. Study Rationale

Insulin resistance, compensatory hyperinsulinemia, and high blood lipid concentrations (prediabetic state) precede overt type 2 DM. Obesity and insulin resistance appear to be key predictors of type 2 DM that may be responsible for pancreatic β-cell incompetence and eventual dysfunction. Dietary modification is key in delaying the progression of the prediabetic state to type 2 DM.

Although the fa/fa Zucker rat does not develop type 2 DM, it is a genetic animal model of the prediabetic state similarly found in humans with obesity, as the model has obesity, altered lipid metabolism and insulin resistance. In addition the fa/fa Zucker rat has leptin resistance as observed in high serum leptin concentrations, a characteristic also observed in many obese humans (reviewed by Tallman and Taylor 1999). An advantage to using a genetic model is the characteristics being studied are already present and do not have to be induced using "artificial environments". For example DIO studies induce obesity, insulin resistance and altered lipid and carbohydrate metabolisms in genetically lean rats, the phenotype of the fa/fa Zucker rat. Therefore, the fa/fa Zucker rat was an excellent *in vivo* model to assess the effects of dietary zinc or n-3 fatty acids on obesity, insulin resistance (hyperinsulinemia), leptin resistance (hyperleptinemia), and lipid metabolism (some serum lipid parameters).

Although some studies have examined the effect of zinc supplementation on the pancreas in diabetic animals, research is limited on the effects of dietary zinc on pancreatic function and pathology both in obesity and insulin resistance, and in type 2 diabetes. Zinc injections provide some protection in pancreas against the β-cell cyto-toxic agent, STZ (Yang and Cherian 1994, and Apostolova et al. 1997), and dietary zinc

supplementation delayed the progression of diabetes in diabetes-prone BB rats (DM 1 model) (Tobia et al. 1998). However, more research is needed to understand how dietary zinc protects the pancreas from free radical damage (e.g. MT-induction). As well, more research is needed to determine whether dietary zinc supplementation will help protect pancreatic function through improved peripheral insulin sensitivity, thus reducing insulin hypersecretion, which may delay or prevent β-cell hypertrophy, hyperplasia and β-cell exhaustion.

Abnormal lipid metabolism is also observed in DM2 and in obesity and insulin resistance. However, research is limited on the effects of dietary zinc on lipid metabolism (e.g. serum lipid values). Zinc deficiency induced in lean rats fed less than 3 ppm zinc were found to be deleterious to lipid metabolism leading to high serum or plasma triglyceride concentrations, higher liver triglyceride synthesis, and fatty livers (Collip 1984). Given that altered zinc metabolism also occurs in DM2 and in obesity, as indicated by hyperzincuria and altered tissue status in DM2 (Kinlaw et al. 1980, Levine et al. 1983, Simon 1998, and Petrolaukis 2000) it is possible that altered zinc status may induce or worsen abnormal lipid metabolism in these conditions. *In vivo* studies have shown improved peripheral insulin sensitivity (Tobia et al. 1998) and improved hyperinsulinemia and hyperglycemia (Bégin-Heick et al. 1985 and Simon 1998) in some DM animal models, thus zinc supplementation may have beneficial effects on blood lipids. Therefore, the first hypothesis was that zinc supplementation will improve or protect pancreatic function, increase MT, and increase insulin sensitivity through lower serum insulin and serum lipid concentrations.

Considerable research has compared the effects of fish oil against other dietary oils on both glucose and lipid metabolism in DM2 and in obesity and insulin resistance.

Although most research agrees that fish oil has certain hypolipidemic effects in both obesity and DM2 (Simopoulos 1999 and Montori et al. 2000), the literature is varied concerning the effects of fish oil on insulin resistance and glucose metabolism (Montori et al. 2000). Diet-induced obesity studies, where high fat diets contained 1/3 of the dietary fat amount as fatty fish oil, improved peripheral insulin sensitivity, as observed in glucose uptake and glucose tolerance tests (Storlein et al. 1987) thus possibly helping pancreas function. *In vitro* pancreas and pancreatic islet studies indicate differing effects of individual FFAs on pancreatic insulin secretion based on chain length and degree of saturation (Opara and Hubbard 1993, Zhou and Grill 1994 and Stein et al. 1997). Therefore, the second hypothesis was that diets high in n-3 fatty acids will improve or protect pancreatic function and increase insulin sensitivity through lower serum insulin and serum lipid concentrations.

Most of the comparison studies have used oil from fatty fish; however, with the exception of a few studies on perilla oil, research is limited on the effects of ALA on serum lipid and insulin concentrations, glycemic control and peripheral insulin sensitivity in both obesity, insulin resistance and type 2 DM in humans or animals. Since ALA functions as a precursor of EPA and DHA and as a source of energy, more research is needed to determine if ALA has similar or contrasting effects to EPA and DHA in normalizing blood lipids, insulin sensitivity, pancreatic β-cell function in insulin resistance, and in type 2 DM, promoting glycemic control. As ALA is a precursor to EPA and DHA, the third hypothesis was diets high in ALA will have a similar protective effect on pancreatic function as diets high in EPA and DHA

Comparing the effects of dietary oils on obesity and insulin resistance is an important first step; however, diets contain a mixture of oils and fatty acids. *In vitro*

studies are able to compare the effects of different fatty acids on a particular cell, tissue, or organ function, such as the *in vitro* studies testing the effects of fatty acids on pancreatic insulin secretion (Zhou and Grill 1994 , Opara and Hubbard 1993, and Stein et al. 1997). The strength of *in vitro* studies is the ability to study the effect of one component (e.g. nutrient) on a particular cellular or tissue function without interactions from other components that normally would be present in an *in vivo* system. However, the strength of *in vitro* experiments can also be a limitation. Artificial environments are created to support tissue life, which limits the knowledge of nutrient or component behavior in a living system with other nutrients, possible interactions and other metabolic activities that commonly occur.

The literature has examples of studies testing the effects of specific fatty acids as esters or as highly purified oils in rats (Mori et al. 1997, Mori et al. 1999, Nobukata et al. 2000, and Minami et al. 2002). Oils used in the rat studies had different percentages of saturated (SAT), monounsaturated (MUFA), and polyunsaturated (PUFA) fatty acids, thus putting into question whether a particular fatty acid group (e.g. long n-3 fatty acids of fish oil) actually influenced the results. In addition, a typical human diet usually consists of more than one oil, with combinations of dietary fatty acids that can be categorized as high in a fatty acid group (e.g. saturated, n-6, n-3) or ratio (e.g. n-6:n-3). Therefore, to compare the effects of very long n-3 fatty acids found in fish oil with long n-3 (ALA) and long n-6 (LA) fatty acids on hyperinsulinemia, hyperlipidemia and pancreatic function, dietary oil mixtures were used to maintain a constant proportion of SAT, MUFA and PUFA.

Therefore, the objectives of this thesis were to investigate the role of dietary zinc

or n-3 fatty acids (with controlled SAT, MUFA and PUFA amounts) on 1) body weight, hyperinsulinemia and hyperlipidemia, and 2) pancreatic β-cell function in the fa/fa Zucker rat, a genetic animal model of obesity and insulin resistance.

To study the effects of dietary zinc and long and very long n-3 fatty acids on pancreatic function, hyperinsulinemia and hyperlipidemia in the fa/fa Zucker rat, this thesis was separated into two studies: the Lipid Study and the Zinc Study. The Zinc Study for this thesis was part of a study for another thesis as outlined by Petroulakis (2000). However, with the exception of the diets, number of animals, and not having the oral glucose tolerance test, the study design was similar for both studies.

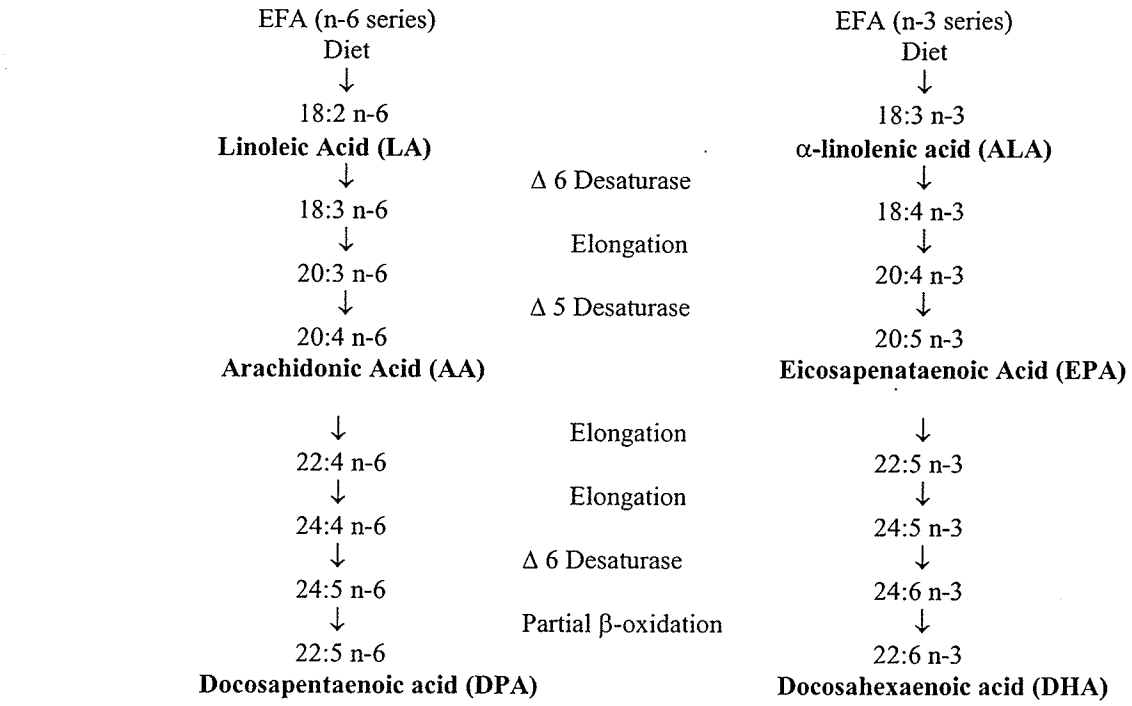
Dietary intervention in both studies started at an early age (five weeks of age) and continued until the rats were about 15 weeks of age. At 15 weeks of age, hyperinsulinemia is at the maximum level (Zucker and Antionades 1972), there is extreme hypertrophy (Shino et al. 1973) and moderate hyperplasia of pancreatic β-cells (Shino et al. 1972, Larsson et al. 1977, and Chan et al. 1998) and the percentage of body lipid has reached a maximum level (Zucker and Antionades 1972). As similarly stated by Petrolaukis (2000) for the Zinc Study, the length of the Lipid Study was chosen as a sufficient period to demonstrate the effects of the different diets. Rationale for the Zinc Study has been described by Petroulakis (2000).

A limitation of many other studies has been the high fat diets (e.g. 20-30% w/w fat). The percent dietary fat used in the Lipid Study was chosen to be at a moderate level (10% w/w). The percent level of fat for the diet was based on the minimal level of fat that caused significant effects in other studies (Bunce et al. 1992, Chicco et al. 1996, and Giron et al. 1999), while protecting the animals from possible lipotoxic effects from

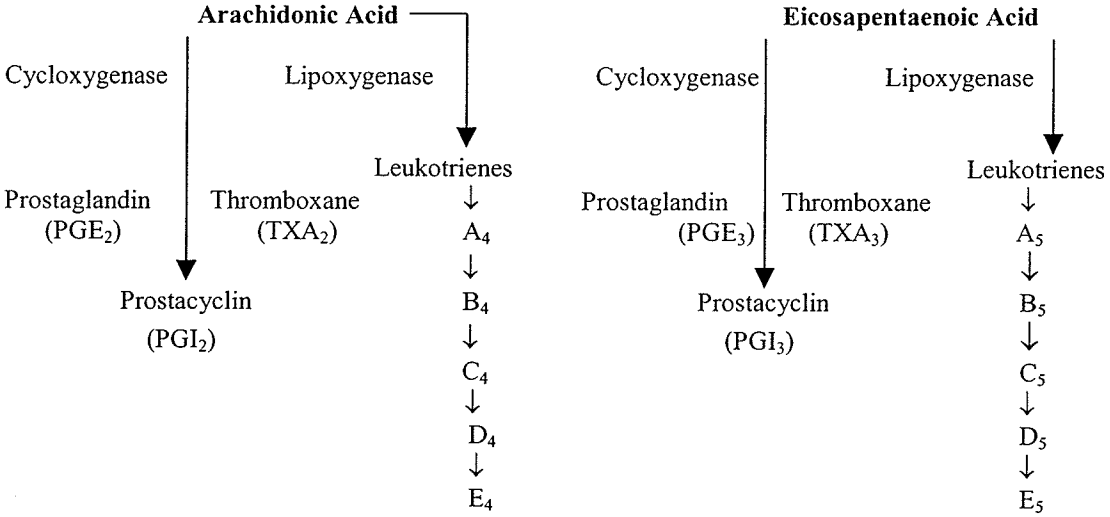
ingesting a high fat diet. Bunce and colleagues (1992) observed normal glucose tolerance tests in 300-day old male BHE/cdb rats (develops abnormal glucose tolerance at age 300 days) that were fed a diet containing 10% (w/w) menhaden oil compared to rats fed diets with either 10% (w/w) beef tallow or corn oil. Chicco and colleagues (1996) observed lower liver and serum triglycerides, total cholesterol, and HDL in male Wistar rats fed diets with 8% (w/w) cod liver oil mixture compared to rats fed diets with 8% (w/w) corn oil. Giron and associates (1999) reported lower plasma triglyceride concentrations in female Wistar rats fed diets with 5% (w/w) olive oil compared to rats fed diets with either 5% (w/w) sunflower or fish oil. All diets in the Lipid Study had 30 ppm zinc with remaining nutrient amounts based on the AIN-93G diet (Reeves et al. 1993).

Dietary oil mixtures were used to compare n-6 versus n-3 PUFAs and long n-3 versus very long n-3 PUFAs (*Table 2*). In all dietary oil mixtures, total amounts of SAT, MUFA and PUFA, as well as the PUFA/SAT ratios were controlled to prevent confounding factors due to the unique fatty acid profiles of the different dietary oils. Names of each diet reflected the principal dietary oil of each diet. The menhaden oil (MO) diet contained primarily menhaden oil (7% w/w), the safflower oil (SO) diet contained primarily safflower oil (7% w/w), and the flax seed oil diet contained primarily flax seed oil (6% w/w). A pair-weighed group fed the SO diet was included to help protect against a potential confounder of weight loss. Weight loss could improve insulin sensitivity and pancreatic function, thus making interpretation of the results difficult. The pair-weighed group was fed the SO diet in an amount to maintain similar body weights to the MO and FXO groups, whichever weighed less.

Similar measurements were made in the Lipid and Zinc Studies with the exception of the oral glucose tolerance test and serum insulin and glucose measured only in the Lipid Study. The oral glucose tolerance test was used to evaluate the effect of n-6 and long or very long n-3 fatty acids on pancreatic function in the fa/fa Zucker rat. Glucose tolerance tests indicate the dynamic pancreatic β-cell function, as well as peripheral insulin sensitivity (Gallwitz 1998). Immunohistochemical analyses for pancreatic insulin and metallothionein localization were also used in both the Zinc and Lipid Studies to investigate the effects of dietary zinc or dietary oil mixtures (n-6, long or very long n-3 fatty acids) on pancreatic function of the fa/fa Zucker rat.



A. "n-6 family" "n-3 family"



B. "2 series eicosanoids" "3 series eicosanoids"

Figure 1. Outline of Pathways of n-6 and n-3 Essential Fatty Acid Metabolism: A. Elongation and Desaturation, and B. Eicosanoid Production (adapted from Opara and Hubbard 1993, Groff et al. 1995, and Sprecher 1999).

Table 1. Characteristics of the fa/fa Zucker Rat During Its Lifecycle.

Weeks of Age	Characteristic Features
2 to 4, as late as 6	- fa/fa trait determined through: hyperinsulinemia[4,6,8] lactescence of blood serum (elevated triglyceride, free fatty acids and LDL levels)[2,8] hyperphagia [1,2]
	- visible and palpable differences in body fat content and shape [1,2]
	- hypertrophy of islet cells[8]
	- hypertrophy and hyperplasia of adipocytes begin[4]
6 to 7	- abnormal oral glucose tolerance test, but normal basal glycemia[11]
	- advanced hypertrophy and marked alterations of islet cells[8,10]
	- most β-cells degranulated[8]
	- moderate β-cell hyperplasia observed[7,8,10]
10 (to 20)	- prominent peripheral insulin resistance[4,11]
12	- albuminuria evident in fa/fa, renal function similar to lean[9]
14	- marked mesangial matrix expansion (before FGS) evident[9]
	- focal glomerulosclerosis (FGS) developed at an early age[9]
15 to 24	- β-cells extremely hypertrophied, degranulated islets, some proliferation of fibrous tissue[8]
15	- hyperinsulinemia reaches maximum level
	- percentage of body lipid and fat cell size maximum[4]
18	- marked albuminuria compared to lean littermates[9]
20	- elevated resting blood glucose after prolonged fasting (normoglycemia at age 26-50 weeks)[3,4,11]
	- abnormal oral glucose intolerance more pronounced[11]
	- indications of β-cell formation from extrainsular cells[7,8]
	- pronounced β-cell hyperplasia (age: 100 to 200 days)[7]
35 to 40	- renal lesions occur (hydronephrosis, kidney stones)[2,5]
52	- hyperinsulinemia disappears[8]
	- islet cells still hypertrophied but β-cells are granulated[8]
	- hyperplasia of β-cells decreases[7]
	- increased fibrous tissue in islets[8]
<52 to 73	- death[5,7,9], usually preceded by weight loss[5] (usually caused by renal failure)[9]

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III. MATERIALS AND METHODS

LIPID STUDY

Animals and Diets

Zucker rats were purchased from Charles River Laboratories (St. Constant, Quebec). The start date for 8 groups of rats was staggered over an eight-week period. Each group consisted of 4 fa/fa and 4 lean 5-week-old male Zucker rats. At 5 weeks of age, fa/fa rats can easily be distinguished from the lean rats by having higher body weights. Upon the arrival of each group, rats were randomly assigned to the dietary groups (*Table 3*), and individually housed in stainless steel wire-bottomed cages. During a 5-day acclimatization period rats were fed the safflower oil mixture diet. After the adaptation period, the rats were weighed and were fed ad libitum the test diets for 9 weeks. To accommodate the oral glucose tolerance testing, the start date of the dietary treatment was arranged to have the fa/fa rats of the group start on the same day and the lean rats start on the next day, or vice versa.

Each diet was nutritionally adequate and was based on the AIN-93G formulation (Reeves et al. 1993), but each diet had 10% (w/w) fat instead of the recommended 7% (w/w) (*Table 4*). The test diets contained the flaxseed oil mixture (FXO) diet, menhaden oil mixture (MO) diet, or safflower oil mixture (SO) diet as the fat source. The FXO diet primarily consisted of flaxseed oil, (6% w/w), with the remaining oils being safflower oil (1% w/w), coconut oil (2% w/w), and canola oil (1% w/w). The MO diet consisted of menhaden oil (7% w/w) and safflower oil (3% w/w). The SO

diet consisted of safflower oil (7% w/w), coconut oil (2% w/w), and canola oil (1% w/w). The fatty acid composition of dietary oil mixtures was determined (*Table 2*) as described in *Extraction and Analysis of Fatty Acids in Diets*. The dietary oil mixtures contained 25.2-26.2% SAT, 18.4-20.5% MUFA and 53.3-55.1% PUFA. The MO oil mixture contained 17.1% EPA plus DHA, the FXO dietary oil mixture contained 35.5% ALA and the SO dietary oil mixture contained 54.1% LA. The n-6 to n-3 ratios for the MO, FXO and SO diets were 1.0, 0.5 and 58.6, respectively, while the PUFA to SAT ratio was close to 2.1 for all groups. Diet was prepared weekly and stored at –20 °C until fed.

In addition to the randomization of rats to each dietary treatment group, one fa/fa rat and one lean rat of each staggered group were designated as pair-weighed (PW) animals. The PW fa/fa or lean rat was fed the SO diet in an amount required to maintain a similar body weight as the lightest fa/fa or lean rat, respectively, of each staggered group.

Throughout the acclimatisation and study periods, the rats were maintained in a controlled environment of 55% humidity, 21-23°C with a 14-hour light, 10-hour dark cycle. Rats were provided with tap water ad libitum, available in acrylic plastic bottles with rubber stoppers and stainless steel sipper tubes. Diet was provided in metal food cups with stainless steel lids. Feed was replaced daily with fresh diet. Lids were replaced with clean lids every second day and food cups were replaced at least once a week. To accommodate the larger size of the rats, lids were not used starting at week 6 of the feeding trial.

Feed intake was weighed daily with diet spillage recorded when necessary. Body weights were recorded weekly.

Animal care procedures were based on the guidelines outlined in the Canadian Council on Animal Care (1993). The University of Manitoba, Fort Garry Campus Protocol Management and Review Committee approved the study protocol.

Oral Glucose Tolerance Test (OGTT)

To test the effect of the different dietary oil mixtures on the response of lean and fa/fa Zucker rats to a glucose load, the rats underwent oral glucose tolerance testing (OGTT) after 8.5 weeks of the dietary treatment. The OGTT provides information on the ability to metabolize a glucose load to pre-challenge blood glucose levels, the time to achieve normal blood glucose concentrations, pancreatic activity during the OGTT through insulin concentrations and indirectly, the extent of peripheral insulin resistance, if present (Gallwitz et al. 1998). The OGTT protocol was based on the technique published by Hem et al. (1998).

Materials

- Razor and brush (Wahl, USA)
- Towels
- Gauze (8 ply, 10 cm X 10 cm, Catalogue No. 2556, Kendall Curity, Mansfield, Massachusetts)
- Timer
- 22 gauge needles (Catalogue No. 305155, Becton Dickinson, Franklin Lakes, New Jersey)
- 1 mL syringes (Catalogue No. 309602, Becton Dickinson)

- Microvettes with clot activator (Catalogue No. 16.440.300, Sarstedt, Nümbrecht, Germany)
- Ice
- Glucose Solution (50 g/ 100 mL or 0.5 g/ 1 mL– Prepared by dissolving 105 g D (+)-Glucose Anhydrous (Sigma, Catalogue No. G-7528, St. Louis, Missouri) in 150 mL of deionized water.)
- Polypropylene microcentrifuge tubes (500 μL, Eppendorf, Catalogue No. 22 36430 8, Germany)

Procedure

OGTTs for each block of fa/fa and lean Zucker rats occurred in the afternoon, 5 days prior to necropsy. One week before OGTT, the rats underwent OGTT training to familiarize the animals with the procedures and to minimize stress during OGTT. Stress could induce the release catecholamines that could influence blood glucose concentrations. As well, rats were handled often throughout the feeding trial to further minimize stress.

OGTT Training

Five hours before OGTT training, food cups were removed from the cages. Prior to training, rats were transferred to plastic cages and were transported to the procedures room. To restrain the rats, each rat was wrapped in a towel with the head covered. One hind leg was grasped and lightly extended. The razor was turned on and was placed against the exposed leg to permit the rat to feel the vibration of the razor. After the other hind leg underwent the same procedure, the rat was fed an aliquot of the glucose solution through a syringe. The fa/fa and lean Zucker rats were

fed 0.75 mL and 0.5 mL of the 50% glucose solution, respectively. The volume amounts were based on the following formula

Glucose amount (g) = Body weight (g) X (0.07 g glucose/100 g of body weight)

Glucose dose (mL) = Glucose amount (g)/0.5 g of glucose/1 mL

Average body weights of control fa/fa and lean Zucker rats from the Zinc Study at ages similar to ages of rats being tested were used in the calculation of glucose volumes used during OGTT training.

After OGTT training, the rats were returned to their original cages and were given access to their food cups.

OGTT

Food cups were removed from the block of fa/fa and lean Zucker rats to be tested, five hours before OGTT. Prior to OGTT, rats were transferred to individual plastic cages and transported to the procedures room. Similar to the OGTT training period, each rat was wrapped in a towel with its head loosely covered and easy access to both hind legs. The towel immobilized the rat and served as a gentle restraint. Each hind leg was shaved with a razor to visualize the location of the saphenous vein of each leg. The baseline (t = 0) blood was collected by locating the saphenous vein of one hind leg. When the vein was clearly visible, the vein was pricked at a 90° angle with a 22 gauge needle. Approximately 300 μL blood was collected in a microvette tube

(Sarstedt, Nümbrecht, Germany) and then placed on ice. Gauze was placed over the wound and some pressure was applied to stop the bleeding.

After the baseline blood sample was collected, the rat drank an aliquot of the glucose solution, provided through a syringe. The volume of glucose solution was based on the calculation described in ***OGTT Training***; however, the most recent body weight (taken 1 to 2 days previously during the weekly weighing) was used in the calculation. Blood collections were repeated at 15, 30 and 60 minutes, after which the rat was returned to its cage and was given access to its food cup.

Once blood was collected for all time points for each animal, the microvettes were placed in a centrifuge (Micromax, International Equipment Company, Needham Heights, Massachusetts) at 4°C and centrifuged at 1500 RPM for 15 minute to separate the serum. Serum was transferred to labelled microcentrifuge tubes, and stored at – 80°C.

Sample Collection

Urine

Urine was collected at weeks 0, 3, 6 and 9 of the study. Rats were weighed and then fasted overnight (12 hours) in polycarbonate metabolic cages (Nalgene, Fisher Scientific) to obtain urine samples that were not contaminated by diet. Rats had free access to tap water in the metabolic cages. Urine was collected in pre-weighed 20 mL polypropylene tubes that were attached to the collecting ducts of the metabolic cages. After the urine collection, the vials were capped, weighed and stored at -20°C.

Tissues

After an overnight fast in the metabolic cages, four rats (all fa/fa or all leans) were terminated at week 9 of the dietary treatment, using CO_2 asphyxiation. Trunk blood was collected in 12 x 75 mm polypropylene tubes (Fisher Scientific), stored on ice and centrifuged at 1290 x g in a centrifuge (Beckman TJ-6) at 4°C for 15 minutes to obtain serum. Serum was separated into aliquots and stored at – 80°C to be analysed for zinc, free fatty acids, triglycerides, leptin, C-peptide, glucose, and insulin concentrations. The tail portion of the pancreas was prepared for immunohistochemical analyses by being immersed into a jar of 10% phosphate-buffered formalin (Fisher Scientific). Organs and tissue were weighed, immediately frozen in liquid nitrogen and stored at - 80°C.

ZINC STUDY

A description of the study and diet protocol for this study can be found in K. Petroulakis' (2000) Master's Thesis. Briefly, 5-week old weanling male fa/fa and lean Zucker rats (28 fa/fa and 28 lean, total=56 rats) were randomised into one of three dietary treatment groups: zinc-deficient (ZD, 5 ppm zinc), zinc control (ZC, 30 ppm zinc) and zinc-supplemented (ZS, 150 ppm zinc). Lean and fa/fa pair-weighed groups fed the ZC diet were also included. Each rat was fed the respective diet for nine weeks. Body weights were measured weekly and feed intake was measured twice weekly. Urine was collected prior to study and at three-week intervals (0, 3, 6 and 9 weeks of study). No oral glucose tolerance tests were performed on these

animals. Similar tissue collection occurred at the end of the 9 week feeding trial. Similar analyses were performed on serum and pancreatic tissue of the rats.

EXTRACTION AND ANALYSIS OF FATTY ACIDS IN DIET

Diet samples from freshly made menhaden oil (MO), safflower oil (SO), and flaxseed oil (FXO) diets were analyzed at the beginning, middle and at the end of the study for percent fat and fatty acid profiles. As well, 24 hour MO, SO, and FXO food cup samples from the animal room were obtained on two separate days of the study.

Fat Extraction

The fat extraction protocol was adapted from Folch et al. (1957). In a 100 mL beaker, two grams of fresh diet or food cup sample were homogenized in 40 mL of 2:1 chloroform:methanol (HPLC grade reagents) using a Polytron Homogenizer (speed 4) for 30 seconds. The homogenate was filtered through Whatman #1 (9 cm) filter paper into a 100 mL graduated cylinder. Both the beaker and filter paper were rinsed with another 10 mL of 2:1 chloroform: methanol. The volume in the graduated cylinder was recorded and 25% of this volume was added as water. The graduated cylinder was stoppered, inverted five times to mix the solutions and allowed to sit overnight.

The following day, the volume of the chloroform (bottom) layer was recorded. The top layer was suctioned off as waste and a calculated volume (explained in the ***Fatty Acid Methylation and Analysis*** section) of the chloroform layer was set aside

for fatty acid analysis. As well, a 20 mL aliquot of the chloroform layer was pipetted into a pre-weighed, 25 mL glass vial and evaporated under nitrogen at 50-70°C for approximately 1 hour or until the vial was dry. The vial was placed in a dessicator overnight and weighed.

The percent fat was calculated as follows

$$\frac{(\text{Wt (g) of vial \& oil}) - (\text{wt (g) of vial})}{2.00 \text{ g sample}} \times \frac{\text{volume (mL) of chloroform layer}}{20 \text{ mL aliquot}} \times 100\% = \% \text{ Fat in sample}$$

Fatty Acid Methylation and Analysis

To determine the fatty acid profile by gas chromatography, the fatty acids in the sample (e.g. oil) are methylated. The methyl ester form makes the fatty acid volatile and easier to analyze using gas-liquid chromatography. The principle of gas-liquid chromatography is that methylated fatty acids are heated to make the fatty acids volatile. The fractionation and eventual detection of each type of fatty acid in the sample is dependent upon on the boiling point of the fatty acid and the nature of the solid adsorbant of the column used in the gas chromatograph (Harris 1987).

Methylation

Solubilization and methylation of the fat extracts followed a procedure adapted from "Advances in Lipid Methodology –Two" (W.W. Christie, editor). An aliquot of the fat extracted (part of the chloroform layer – see ***Fat Extraction*** section) from the diet or food cup sample was used for fatty acid analysis using gas-liquid chromatography. Determination of the aliquot volume needed for fatty acid analysis

was based on the volume that would provide 10 mg of oil. The volume required was calculated as follows

2.00 g diet X 10% oil (by wt in diet) = 0.2 g or 200 mg oil in diet.

$$\frac{10 \text{ mg} \times \text{total volume (mL) of chloroform layer}}{200 \text{ mg}} = \text{volume (mL) of chloroform needed for fatty acid analysis}$$

The chloroform aliquot was placed into an 8 mL screw top tube that was then placed under nitrogen and in a 35°C water bath to evaporate the solvent. After evaporation, 1 mL toluene (HPLC grade) was added to the tube and vortexed for 10 seconds to solubilize the fat extract. To methylate the fat sample, a 1.2 mL aliquot of 3 N methanolic hydrochloric acid was added, after which the tube was tightly capped, vortexed for 10 seconds and placed in an 80°C oven.

After 60 minutes, the tubes were removed from the oven and cooled to room temperature. The cooled tubes received 1 mL water and 1 mL hexane (HPLC grade), and were vortexed for 20 seconds and centrifuged (Model CS, International Equipment Company, Boston, Massachusetts, USA) at ¾ speed (2000 rpm) for 4 minutes. The hexane (top) layer was transferred to a clean 8 mL tube. To the hexane layer, 2 mL water was added, the tube was capped, vortexed for 20 seconds, and centrifuged at ¾ speed for 4 minutes. The hexane (top) layer was transferred to GC vials for fatty acid analysis.

Fatty Acid Analysis

The prepared samples were analysed at the using a Varian Star 3400 Gas Chromatography System (Georgetown, Ontario) with a flame ionisation detector and

a DB-225MS capillary column (30 m x 0.25 mm I.D. with 0.25 μm film thickness). The injector temperature was 250 °C, the detector temperature was 300°C and the column was programmed from 70 to 220 °C.

SERUM GLUCOSE

Glucose concentrations of serum from the OGTT was determined using an enzymatic colorimetric assay for glucose or the glucose oxidase method. This procedure is based on two coupled enzymatic reactions: glucose oxidase (Reaction I) and peroxidase (Reaction II)

I. Glucose + H_2O + O_2 → Gluconic Acid + H_2O_2

II. H_2O_2 + o-Dianisidine (colorless)→ Oxidized o-Dianisidine (brown color)

The intensity of the brown color measured at 425-475 nm is proportional to the glucose concentration in the serum sample.

A Glucose Assay Kit from Sigma Diagnostics (Procedure #510-A) was used. The kit contained the following items that were used to prepare the working solutions:

1. PGO Enzymes (Catalog #510-6)
2. o-Dianisidine Dihydrochloride (Catalog #510-50)
3. Glucose Standard Solution (Catalog #635-100, 100 mg/dl (5.56 mmol/L) in 0.1% benzoic acid solution)

- The enzyme solution was prepared by adding contents of 1 capsule of PGO enzymes to 100 mL deionized water in an amber bottle.
- The color reagent solution was prepared by the reconstitution one vial of o-Dianisidine Dihydrochloride with 20 mL deioinized water.
- The combined enzyme-color reagent solution was prepared by combining 100 mL enzyme solution with 1.6 mL color reagent solution.

Procedure

A glucose standard curve (0-15 mg/dL) was prepared by diluting the glucose standard solution with deionized water. Serum samples initially were diluted 10 fold to determine concentration based on the standard curve and to determine whether further dilutions were required. Dilutions for OGTT serum were between 10 to 25 fold, for fa/fa and lean Zucker rats.

Twenty microliter aliquots of buffer blanks, standards or samples were pipetted in triplicate into 96 well polystyrene microtiter plates (Costar 9017 EIA/RIA plate). A two hundred microliter aliquot of the combined enzyme-color reagent solution was pipetted into each well using a multi-channel pipette. The side of the microplate was tapped six times to mix the contents of the wells. The microplate was then covered with foil and incubated at room temperature (18-26°C) for 45 minutes. Absorbances (A) were read on the plate reader (SpectraMax 340, Molecular Devices Corporation, Sunnyvale, California) at 450 nm and the concentration for each sample was calculated using the microplate software (SOFTmax Pro, version 1.2.0) provided

by the company that based its calculations on the glucose standard curve. The concentrations were adjusted for dilutions and converted to mM.

SERUM INSULIN

The protocol for the determination of the insulin concentration in serum from the Lipid study was adapted from the rat insulin radioimmunoassay (RIA) protocol outlined by Linco Research, Inc. (St. Charles, MO). The protocol for the determination and calculations of serum insulin concentrations of the Zinc Study can be found in the Master's thesis of Kathy Petrolaukis (2000). The principle of the radioimmunoassay is that labelled and unlabeled insulin compete for the limited binding sites available on the antibody (anti-rat insulin antibody). The amount of labelled insulin bound to the antibody is inversely proportional to the concentration of unlabeled insulin in the serum.

A RIA kit (kit lot # SRIR-108, Linco Research, Inc.) was used for the determination of insulin. The kit contained the following reagents:

- Assay buffer (0.05 M Phosphosaline, pH 7.4, 0.025 M EDTA, 0.1% Sodium Azide, and 1% RIA grade BSA)
- Rat insulin antibody (produced in guinea pig)
- ^{125}I-Rat insulin label (<3 μCi, <111kBq)
- Label hydrating buffer, which contained normal guinea pig IgG as a carrier
- Rat insulin standards (0.02, 0.05, 0.1, 0.2, 0.5, 1.0 ng/ mL)
- Two quality controls
- Precipitating reagent.

Procedure

Day 1

With the exception of the total count tubes, assay buffer was pipetted into borosilicate glass tubes (12 X 75 mm): 300 μL of assay buffer was pipetted into the non-specific binding (NSB) tubes, 200 μL was pipetted into the reference (Bo) tubes and 100 μL was pipetted into each quality control and sample tube. One hundred microliters of standards, quality controls, or diluted samples were pipetted into appropriate tubes. Lean Zucker rat serum was diluted by a factor of 5, while fa/fa Zucker rat serum was diluted by a factor of 100.

With the exception of the total count and NSB tubes, 100 μL aliquots of rat insulin antibody were pipetted into each tube. All tubes were vortexed, covered, placed in a humid chamber and left to incubate overnight at 4 °C.

Day 2

Each tube received 100 μL of ^{125}I-rat insulin. Tubes were vortexed, covered, returned to the humid chamber and incubated overnight at 4 °C.

Day 3

With the exception of the total count tubes, 1.0 mL aliquots of cold precipitating reagent (4 °C) were pipetted into all tubes. The tubes were then vortexed, covered, returned to the humid chamber and left to incubate at 4°C for approximately 20 minutes. All tubes, except the total count tubes, were centrifuged at 2000 x g in a centrifuge (Beckman TJ-6) for 40 minutes to obtain a firm pellet. The internal

temperature of centrifuge was controlled at 4 °C. Except for the total count tubes, pellets for each tube were isolated by immediately decanting the supernatant fraction and then draining each tube for 20 seconds to remove excess liquid. Tubes were placed into a gamma counter (Beckman Gamma 8000, Scientific Instruments, Irvine, California) and each tube was counted for 10 minutes. The counts were measured in counts per minute (CPM).

Calculations

Duplicate counts were averaged for total counts, NSB, B_o, quality controls, standard and samples. The average NSB count was subtracted from all tubes except the total count tubes. Percentage of maximum binding or $\%B/B_o$ for each standard and sample were calculated. GraphPad PRISM, version 2.0 (GraphPad Software, San Diego, California) was used to calculate the insulin concentration for each sample. This software program plotted a standard curve by plotting each standard $\%B/B_o$ on the y-axis against its known concentration on the x-axis. The insulin concentration of each sample was then determined by interpolating the concentration value from the standard curve. Sample concentration values calculated by the Prism software program were then multiplied by the appropriate dilution factor. The insulin concentration was expressed as nanograms per milliliter (ng/mL). Sample concentrations were accepted if the average counts were found to be within the standard curve.

The assay was considered valid if the concentrations of the quality control samples were within 2 standard deviations of the expected concentration range listed

by the company. Average CPM for each sample was accepted if the difference between the duplicates was less than 10% CV.

SERUM C-PEPTIDE

The protocol for the determination of C-peptide concentration in serum for both Zinc and Lipid studies was adapted from the rat C-peptide RIA protocol outlined by Linco Research, Incorporated. The principle of the RIA is that labelled and unlabeled C-peptide compete for the limited binding sites available on the antibody (anti-rat C-peptide antibody). The amount of labelled C-peptide bound to the antibody is inversely proportional to the concentration of unlabeled C-peptide in the serum.

A RIA kit (kit lot # RCPR-066, Linco Research, Inc. (St. Charles, MO.) was used for the determination of C-peptide. The kit contained the following reagents:

- Assay buffer (0.05 M Phosphosaline, pH 7.4, 0.025 M EDTA, 0.1% Sodium Azide, and 1% RIA grade BSA)
- Rat C-peptide antibody (produced in guinea pig)
- ^{125}I-Rat C-peptide label (<3 μCi, <111kBq)
- Label hydrating buffer, which contained normal guinea pig IgG as a carrier
- Rat C-peptide standards (25, 50, 100, 200, 400, 800, 1600 pM)
- Two quality controls
- Precipitating reagent.

Procedure

Day 1

With the exception of the total count tubes, assay buffer was pipetted into borosilicate glass tubes (12 X75 mm); 300 μL of assay buffer was pipetted into the NSB tubes, 200 μL was pipetted into the B_0 and 100 μL was pipetted into each quality control and sample tube. One hundred microliters of standards, quality controls, or diluted samples were pipetted into appropriate tubes, in duplicate. Lean Zucker rat serum of both studies was diluted by a factor of 10, while fa/fa Zucker rat serum of both studies was diluted by a factor of 40. With the exception of the total count and NSB tubes, 100 μL aliquots of rat C-peptide antibody were pipetted into each tube. All tubes were vortexed, covered, placed in a humid chamber and left to incubate overnight at 4 °C.

Day 2

Each tube received 100 μL of ^{125}I-rat C-peptide. Tubes were then vortexed, covered, returned to the humid chamber and incubated overnight at 4 °C.

Day 3

With the exception of the total count tubes, 1.0 mL aliquots of cold precipitating reagent (4 °C) were pipetted into all tubes. The tubes were vortexed, covered, returned to the humid chamber and left to incubate at 4°C for approximately 20 minutes.

All tubes, except the total count tubes, were centrifuged at 2000 x g in a centrifuge (Beckman TJ-6) for 40 minutes to obtain a firm pellet. The internal temperature of centrifuge was controlled at 4 °C. Except for the total count tubes, pellets for each tube were isolated by immediately decanting the supernatant fraction and draining each tube for 20 seconds to remove excess liquid. Tubes were placed into a gamma counter (Beckman Gamma 8000, Scientific Instruments, Irvine, California) and each tube was counted for 10 minutes. The counts were measured in CPM.

Calculations

C-peptide concentration calculations are similar to the calculations outlined in the calculation section of ***Serum Insulin***. C-peptide concentration was expressed as picomoles per liter (pM).

SERUM LEPTIN

The protocol for the determination of the leptin concentration in serum for both the Zinc and Lipid Studies was adapted from the rat leptin RIA protocol outlined by Linco Research, Inc. (St. Charles, MO.). The principle of the RIA is that labelled and unlabeled leptin compete for the limited binding sites available on the antibody (anti-rat leptin antibody). The amount of labelled leptin bound to the antibody is inversely proportional to the concentration of unlabeled leptin in the serum.

A RIA kit (kit lot # RLR-1122, Linco Research, Inc.) was used for the determination of leptin. The kit contained the following reagents:

- Assay buffer (0.05 M Phosphosaline, pH 7.4, 0.025 M EDTA, 0.1% Sodium Azide, and 1% RIA grade BSA)
- Rat leptin antibody (produced in guinea pig)
- ^{125}I-Rat leptin label (<3 μCi, <111kBq)
- Label hydrating buffer, which contained normal guinea pig IgG as a carrier
- Rat leptin standards (0.5, 1, 2, 5, 10, 20, 50 ng/ mL)
- Two quality controls
- Precipitating reagent.

Procedure

Day 1

With the exception of the total count tubes, assay buffer was pipetted into borosilicate glass tubes (12 X 75 mm): 300 μL of assay buffer was pipetted into the NSB tubes, 200 μL was pipetted into the Bo tubes and 100 μL was pipetted into each quality control and sample tubes.

One hundred microliters of standards, quality controls, or diluted samples were pipetted into appropriate tubes, in duplicate. Lean Zucker rat serum from both studies was undiluted, while fa/fa Zucker rat serum from the Zinc and the Lipid studies were diluted by a factor of 25 and 15.625, respectively.

With the exception of the total count and NSB tubes, 100 μL aliquots of rat leptin antibody were pipetted into each tube. All tubes were vortexed, covered, placed in a humid chamber and left to incubate overnight at room temperature.

Day 2

Each tube received 100 µL aliquots of ^{125}I-rat leptin. Tubes were vortexed, covered, returned to the humid chamber and incubated overnight at room temperature.

Day 3

With the exception of the total count tubes, 1.0 mL aliquots of cold precipitating reagent (4 °C) were pipetted into all tubes. The tubes were vortexed, covered, returned to the humid chamber and left to incubate at 4°C for approximately 20 minutes. All tubes, except the total count tubes, were centrifuged at 2000 x g in a centrifuge (Beckman TJ-6) for 40 minutes to obtain a firm pellet. The internal temperature of centrifuge was controlled at 4 °C. Except for the total count tubes, pellets for each tube were isolated by immediately decanting the supernatant fraction and draining each tube for 15 seconds to remove excess liquid. Tubes were placed into a gamma counter (Beckman Gamma 8000, Scientific Instruments, Irvine, California) and each tube was counted for 10 minutes. The counts were measured in CPM.

Calculations

Leptin concentration calculations are similar to the calculations outlined in the calculation section of ***Serum Insulin***. Leptin concentration was expressed as nanograms per milliliter (ng/mL).

SERUM TRIGLYCERIDE

Triglyceride analysis was performed using an enzymatic colorimetric assay kit from Diagnostic Chemicals Limited (Catalogue #: 210-75). The protocol used for this analysis was previously adapted for a microplate reader (SpectraMax 340, Molecular Devices Corporation, Sunnyvale, California). The procedure involves lipase (Reaction I), glycerol kinase (Reaction II), L-α-glycerol phosphate oxidase (Reaction III) and peroxidase (Reaction IV).

I. Triglycerides $\rightarrow$ Glycerol + Fatty Acids

II. Glycerol + ATP $\rightarrow$ Glycerol-1-phosphate + ADP

III. Glycerol-1-phosphate $\rightarrow$ H_2O_2 + Dihydroxyacetone Phosphate

IV. 4-Aminoantipyrine + H_2O_2 + DHBS $\rightarrow$ Quinoneimine Dye + HCl + $2H_2O$

(Colourless) (Red colour absorbs at λ = 515 nm)

DHBS = 3,5-dichloro-2-hydroxy-benzenesulfonic acid

The intensity of the red color measured at 515 nm is proportional to the triglyceride concentration in the serum sample.

Procedure

Different standard concentrations (0.5 mM, 1.0 mM, 2.0 mM) were prepared by diluting a triglyceride glycerol standard solution (equivalent to 2 mM triolein) which was provided in the assay kit. The standard concentrations were prepared by diluting the standard solution with 0.9% sodium chloride solution. The 0.9% sodium chloride solution was prepared by dissolving 2.25 g of sodium chloride (Fisher, Catalogue #:

BP358-212) in 250 mL of deionized water. A reagent blank (0 mM) was prepared using 0.9% sodium chloride solution. Serum samples were also diluted with 0.9% sodium chloride to ensure their values were within the standard curve. Lean Zucker rat serum samples had a dilution factor of 4 while most fa/fa Zucker rat serum samples had a dilution factor of 10. All samples and standards were kept on ice.

Triplicates of 10 μL aliquots of prepared standard, blank or serum sample were pipetted into a 96 well polystyrene plate (Costar 9017, EIA/RIA plate).
Ten μL of prepared standard, blank or serum sample followed by 100 μL reagent was pipetted into each well. The plate was incubated at 25°C for 15 minutes. After adding 140 μL deionized water, the absorbance (A) was read at 515 nm (Spectra Max 340, Molecular Devices Corporation, Sunnyvale, California). Measurements were accepted if the standard curve r^2 was greater than 0.9 and the %CV was less than 10 for the triplicate readings of each standard, blank and serum sample. Triglyceride concentrations of each serum sample was calculated using the microplate software (SOFTmax Pro, version 1.2.0, Molecular Devices Corporation, Sunnyvale, California) that based its calculation on the standard curve. The concentrations were adjusted for dilutions:

Triglyceride (mM) = Concentration from the Standard Curve X Dilution Factor

SERUM FREE FATTY ACID

Free fatty acid concentration was determined using the Free Fatty Acids, Half-micro Test assay kit from Roche Diagnostics (Catalogue #: 1 383 175,), an enzymatic

colorimetric assay. The protocol used was adapted for a microplate reader (Spectra Max 340, Molecular Devices Corporation, Sunnyvale, California). The principle of the assay is based on three enzymatic reactions using acyl-CoA synthetase (Reaction I), acyl-CoA oxidase (Reaction II) and peroxidase (Reaction III).

I. Free Fatty Acids + CoA + ATP $\rightarrow$ Acyl – CoA + AMP + Pyrophosphate

II. Acyl – CoA + O_2 $\rightarrow$ Enoyl – CoA + H_2O_2

III. H_2O_2 + 4 - AA + TBHB $\rightarrow$ Red Dye + $2H_2O$ + HBr

(Colourless) (Red colour absorbs at λ= 546 nm)

4-AA = 4 – Aminoantipyrine

TBHB = 2,4,6 – tribromo - 3 – hydroxybenzoic acid

The intensity of the red colour is proportional to the original free fatty acid concentration in the serum sample.

Effectiveness and sensitivity of each kit was tested by measuring the concentration of a palmitic acid standard solution by a spectrophotometer (Spectronic 3000 Array, Milton Roy Company, USA). The palmitic acid standard solution was prepared by dissolving 0.0379 g palmitic acid (Sigma Chemicals, Catalogue # P0500) in 6 mL of warm (35 – 40 °C) ethanol (Fisher Scientific, Catalogue #: A962-4). The standard solution was diluted to 1.478 mM using a 6% Triton X-100 solution. One hundred mL of 6% Triton X-100 solution was made by dissolving 6.0 g of Triton X-100 (Sigma Chemical Company, Catalogue #: T-8787) in deionized water (30 – 40 °C).

The free fatty acid concentration of an in-house control rat serum sample was also determined using the same spectrophotometer. This sample was obtained from a previous study that used Sprague-Dawley rats.

Procedure

Milton Roy Spectrophotometer

For the spectrophotometer assay, 1.00 mL of reaction mixture A (solution I) was added to 1 mL polypropylene cuvettes. Reaction mixture A contained ATP, coenzyme A, acyl-CoA-synthetase, peroxidase, ascorbate oxidase, 4-aminoantipyrine, potassium phosphate buffer, tribromohydroxybenzoic acid, magnesium chloride and stabilisers.

Aliquots (0.05 mL) of either double deionized water (reagent blank), palmitic acid standard, or in-house serum control were pipetted into individual cuvettes. Cuvettes were mixed and were allowed to stand at room temperature (22°C – 23°C). After about 10 minutes, 0.05 mL aliquots of N-ethyl-maleinimide-solution (solution II) were added to each cuvette. N-ethyl-maleinimide-solution contained N-ethyl-maleinimide and stabilisers. Cuvettes were mixed and absorbances of the solutions (A_1) were read at 546 nm.

Aliquots (0.05 mL) of reaction mixture B (solution III) were pipetted into each cuvette. Reaction mixture B contained acyl-CoA-oxidase, acyl-CoA-oxidase dilution solution and stabilisers. Each cuvette was mixed and incubated at room temperature. After 20 minutes, absorbances of the solutions (A_2) were read at 546 nm.

Calculations for serum free fatty acid concentrations were as follows

$$C = \frac{V}{\varepsilon \times d \times v} \times \Delta A \text{ [mM sample solution], where}$$

V = final volume (mL), (1.15 mL)

v = sample volume (mL), (0.050 mL)

d = light path (cm), (1 cm for Spectronic 3000 spectrophotometer)

ε = absorption coefficient of the dye at 546 nm: 19.3 x $(1 \times mmol^{-1} \times cm^{-1})^3$

ΔA = absorbance difference of the sample minus the absorbance difference of the blank, $(A_2 - A_1)_{sample} - (A_2 - A_1)_{reagent\ blank}$

A_1 = absorbance at time 0 before the addition of the reaction mixture B (solution III).

A_2 = absorbance 20 minutes after the addition of reaction mixture B (solution III).

Therefore, free fatty acid concentrations that were analyzed by the spectrophotometer were calculated using the following formula

$$C = \frac{1.15\ mL}{19.3 \times (1 \times mmol^{-1}cm^{-1})^3 \times 0.050\ mL \times 1\ cm} \times \Delta A$$

or

C = 1.192 x ΔA (mmol free fatty acids/L serum)

Microplate Reader

After testing each new kit, reagent volumes were decreased proportionally so the samples could be analyzed using the microplate reader (0.230 mL final volume). Aliquots (0.200 mL) of reaction mixture A were pipetted into each well of a Costar EIA/RIA polystyrene 96 well plate. Triplicate aliquots (0.010 mL) of either

deionized water (reagent blank), in-house serum control sample or unknown sample were pipetted into the 96 well plate. The 96 well plate was placed into the microplate reader, mixed for 30 seconds, and incubated at room temperature (22°– 23°C).

After approximately 10 minutes, the 96 well plate was removed from the microplate reader. Aliquots (0.010 mL) of N-ethyl-maleinimide-solution were added to each well. The 96 well plate was placed in the microplate reader, mixed for 30 seconds and absorbances (A_1) were read at 546 nm. The 96 well plate was removed from the microplate reader and 0.010 mL aliquots of reaction mixture B were added to each well. The 96 well plate was placed into the microplate reader, mixed for 30 seconds and incubated at room temperature (22°C– 23°C). After 20 minutes, the 96 well plate was mixed for 30 seconds, and absorbances (A_2) were read at 546 nm.

Calculations for serum free fatty acid concentrations were as follows

$$C = \frac{V}{\varepsilon \times d \times v} \times \Delta A \text{ [mM sample solution], where}$$

V = final volume (mL), (0.230 mL)

v = sample volume (mL), (0.010 mL)

d = light path (cm), (0.53326 cm for the microplate reader)

ε = absorption coefficient of the dye at 546 nm: $19.3 \times (1 \times \text{mmol}^{-1} \times \text{cm}^{-1})^3$

ΔA = absorbance difference of the sample minus the absorbance difference of the blank, $(A_2 - A_1)_{sample} - (A_2 - A_1)_{blank}$

A_1 = absorbance at time 0 before the addition of reaction mixture B.

A_2 = absorbance 20 minutes after the addition of reaction mixture B.

Therefore, free fatty acid concentrations that were analyzed by the microplate reader, using Costar EIA/RIA polystyrene 96 well plates, were calculated using the following formula:

$$C = \frac{0.230 \text{ mL}}{19.3 \times (1 \times \text{mmol}^{-1}\text{cm}^{-1})^3 \times 0.010 \text{ mL} \times 0.53326 \text{ cm}} \times \Delta A$$

or

$C = 2.2347 \times \Delta A$ (mmol free fatty acids/L serum)

ZINC CONCENTRATIONS

Zinc concentrations of serum, pancreata, femurs and diets were determined using atomic absorption spectrophotometry. Serum samples were diluted with deionized water by a dilution factor of 10, and were directly analyzed. Pancreata, femurs and diet were subjected to acid digestion prior to zinc analyses.

The acid digestion protocol was based on the technique outlined in Clegg et al. (1981). Approximately 0.5 g of each diet and approximately 0.3 g wet weight of each pancreas was used. One femur of each animal was cleaned of skin and musculature, and the wet weight was recorded.

Both pancreatic portions and femurs were dried for 48 hours in an 85°C drying oven and dry weights were recorded. Each dried tissue and diet samples were placed into a dry, glass test tube that was previously acid washed (30% nitric acid). Bovine liver reference (200 mg/25 mL; ref. # 1477b, U.S. Department of Commerce, National Institute of Standards and Technology, Gainsburg, Maryland, USA) samples were used as quality controls. Each test tube containing dried tissue and blanks received a 1 mL aliquot of 70% nitric acid (trace element grade, Fisher Scientific,

Napean, Ontario). Diet and quality control samples received 2 mL of 70% nitric acid. Each test tube was covered with an acid washed glass marble. Digestion occurred initially at room temperature for 2 hours followed by a 85°C for 48 hours in a dry bath heating block. All pancreas and quality control samples were diluted to 25 mL, all femur samples were diluted to 250 mL and all diet samples were diluted to 10 mL.

Diluted samples were analyzed using an atomic absorption spectrophotometer (Varian Spectra AA-30 Spectrophotometer, Georgetown, Ontario) and zinc standards (0.1-1 ppm) were prepared from a zinc absorption standard (1000 ppm, #H595-01 Mallinckrodt, Paris, Kentucky). Zinc concentration of each sample was calculated as follows:

Pancreas, femur and diet zinc

$$\text{Zinc } (\mu g/g \text{ dry weight}) = \frac{\text{Sample zinc concentration } (\mu g/mL) \text{ X dilution volume } (mL)}{\text{Dry weight of sample } (g)}$$

Serum zinc

$$\text{Zinc } (\mu g/mL) = \text{Sample zinc concentration } (\mu g/mL) \text{ X dilution factor}$$

LOCALIZATION OF PANCREATIC METALLOTHIONEIN USING IMMUNOHISTOCHEMISTRY

The indirect immunoperoxidase technique was used to localize pancreatic MT. Formalin-fixed pancreatic tissue (tail) was embedded in paraffin, cut into 5 μm sections and two tissue sections of each pancreas sample were mounted on fixative-

coated slides by the Pathology Lab at the Health Sciences Centre (Winnipeg, Manitoba). Serial tissue sections were obtained for each rat to compare MT and insulin immunostaining. Tissue sections were deparaffinized and rehydrated using xylene and a graded series of ethanol to water, respectively. Endogenous peroxidase activity of the tissues was blocked by immersing the slides in a fresh 3% hydrogen peroxide bath for 10 minutes and in a PBS (0.15 M NaCl, 10 mM phosphate; pH 7.35) bath for 5 minutes.

To block nonspecific binding, each tissue section was incubated with 250 μL of 20% normal goat serum (DAKO Corporation, Carpinteria, California) in a humid chamber for 90 minutes at room temperature. After the incubation, the normal goat serum was tapped and blotted off each slide. To identify MT, one tissue section of each slide received a 250 μL aliquot of a 1:50 dilution of monoclonal mouse anti-MT antibody (clone E9; DAKO). The other tissue section received a 250 μL aliquot of PBS (negative control). Slides were placed in a humid chamber at 4°C for 21 hours.

Following the primary (MT) antibody incubation, slides were washed well with PBS buffer from a wash bottle and placed into a PBS buffer bath for 5 minutes. Each tissue section received a 250 μL aliquot of prediluted DAKO Envision System goat anti-mouse/anti-rabbit peroxidase labeled polymer (secondary antibody) and each slide was placed in a humid chamber for 45 minutes at room temperature. Slides were washed with PBS buffer and placed into a PBS bath for 5 minutes. To visualize the reaction product, each tissue section was incubated with a 250 μL aliquot of diaminobenzidine tetrahydrochloride (DAB; Polysciences, Inc., Warrington, Pennsylvania) in 10 mL PBS containing 30 μL of 3% hydrogen peroxide for 5

minutes at room temperature. The unreacted DAB was washed off with deionized water after the incubation time. To aid in the visualization of the morphology of the tissue sections, slides were counterstained with 3% hematoxylin for 1 minute. Slides were rinsed with tap water, dehydrated in graded concentrations of ethanol, cleared in xylene and mounted with cover slips using Permount.

Positive staining pattern for MT was confirmed using pancreatic tissue sections from a zinc supplemented (600 ppm) Sprague-Dawley rat as the positive control. Three different negative controls were used to confirm the specificity of staining: omission of the MT primary antibody from the aliquot (PBS buffer only), incubation of pancreatic tissue from a MT null mouse with MT primary antibody, and substitution of anti-MT antibody with mouse IgG1, kappa (clone DAK-GO1; DAKO).

LOCALIZATION OF INSULIN USING IMMUNOHISTOCHEMISTRY

Insulin was localized in pancreatic tissue sections using the avidin/strepavidin immunostaining method. This detection system uses a primary antibody that localizes the target protein (insulin) similar to indirect immunostaining. The distinguishing feature is the avidin/strepavidin system uses a biotinylated secondary antibody and an enzyme, such as horseradish peroxidase, conjugated to another (tertiary) antibody, such as avidin or strepavidin to reduce a chromagen (e.g. DAB) to visually detect the target protein.

Procedure

Similar to tissue preparation for MT staining, serial tissue sections were cut from formalin-fixed, paraffin-embedded pancreatic tail sections. Following deparaffinization, rehydration, and quenching of endogenous peroxidase activity, each tissue section was incubated with a 250 μL aliquot of CYTO Q Background Buster (Innovex Biosciences, Richmond, California) in a humid chamber for 20 minutes to block non-specific binding.

After the incubation, the Background Buster was tapped and blotted off each slide. To identify insulin, one tissue section of each slide received a 250 μL aliquot of a 1:50 dilution of monoclonal mouse anti-insulin antibody (clone E2/E3; Innovex Biosciences). The other tissue section received a 250 μL aliquot of PBS (negative control). Slides were placed in a humid chamber at room temperature for 20 minutes. Following the incubation, slides were briefly rinsed with PBS buffer from a wash bottle and placed in a PBS bath for 5 minutes. Each tissue section received a 250 μL aliquot of the secondary linking antibody (Stat-Q kit, Catalog # NB314KLD, Innovex Biosciences) and each slide was placed in a humid chamber for 10 minutes at room temperature. Slides were rinsed briefly with PBS buffer from a wash bottle and placed in a PBS bath for 5 minutes. Each tissue section received a 250 μL aliquot of peroxidase-strepavidin label (tertiary antibody) (Stat Q kit, Innovex Biosciences) and each slide was placed in a humid chamber for 10 minutes at room temperature. The slides were rinsed briefly with PBS buffer from a wash bottle and placed in a PBS bath for 5 minutes. To visualize the reaction product, each tissue section was incubated with a 250 μL aliquot of DAB substrate solution (Stat Q kit, Innovex

Biosciences) for 5 minutes at room temperature. The unreacted DAB was washed off with deionized water after the incubation time. To aid in the visualization of the morphology of the tissue sections, slides were counterstained with 3% hematoxylin for 1 minute. Slides were rinsed with tap water, dehydrated in graded concentrations of ethanol, cleared in xylene and mounted with cover slips using Permount.

Positive staining pattern of insulin was confirmed using pancreatic tissue sections from a Sprague-Dawley rat. Two different negative controls were used to confirm the specificity of staining: (1) omission of the anti-insulin antibody (PBS buffer only) and (2) substitution of the anti-insulin antibody with mouse IgG1, kappa (clone DAK-GO1; DAKO).

STATISTICAL ANALYSIS

A two-way analysis of variance (ANOVA) was used to determine main effects for genotype, dietary oil mixture, and genotype x dietary oil mixture interaction in the Lipid Study and main effects for genotype, zinc, and genotype x zinc interaction in the Zinc Study. Insulin data were log-transformed for the ANOVA. Repeated measures analysis was also performed on log-transformed OGTT data. If main effects of diet were observed, the Duncan's Multiple Range test was used for means testing. All differences were considered significant at $P \leq 0.05$.

Table 2. Fatty Acid Composition of Dietary Fat Mixtures – Lipid Study[1,2]

	MO 7% Menhaden 3% Safflower	SO 7% Safflower 2% Coconut 1% Canola	FXO 6% Flax 1% Safflower 2% Coconut 1% Canola
C8:0	0	1.69	1.69
C10:0	0	1.17	1.17
C12:0	0.07	8.16	8.16
C14:0	5.28	4.00	3.97
C14:1	0.04	0	0
Pufa[3]	0.36	0	0
C15:0	0.49	0	0
C16:0	14.71	7.84	6.92
C16:1 n9	0.34	0	0
Pufa	0.15	0	0
C16:1 n7	7.03	0.10	0.09
Pufa	1.46	0.01	0.01
C17:0	1.10	0.16	0.14
C17:1	0.25	0	0
Pufa	0.78	0.01	0.01
C18:0	3.12	2.63	3.08
C18:1 n9	9.23	17.14	18.35
C18:1 n7	2.33	0.76	0.77
Pufa	0.16	0	0
C18:2 n6	23.16	54.11	19.15
C18:3 n6	0.41	0	0
Pufa	0.35	0.14	0.28
C18:3 n3	1.13	0.89	35.50
C18:4 n3	2.53	0.04	0.01
Poly	0.12	0.12	0.12
C20:0	0.24	0.31	0.19
C20:1 n9	0.85	0.27	0.17
C20:1 n7	0.20	0	0
Poly	0.20	0.01	0.01
C20:2 n6	0.15	0.01	0.01
C20:3 n6	0.15	0	0
C20:4 n6	0.50	0	0
Pufa	0.11	0	0
C20:3 n3	0.17	0	0
C20:4 n3	1.25	0	0
C20:5 n3	8.00	0	0
C22:0	0.17	0.20	0.11
C22:1 n9	0.02	0.02	0.02
Pufa	0.25	0	0
C21:5 n3	0.43	0	0

Table 2. Fatty Acid Composition of Dietary Fat Mixtures – Lipid Study[1,2](Cont'd)

	MO 7% Menhaden 3% Safflower	SO 7% Safflower 2% Coconut 1% Canola	FXO 6% Flax 1% Safflower 2% Coconut 1% Canola
Pufa	0.67	0	0
C22:5 n3	1.61	0	0
C22:6 n3	9.15	0	0
C24:0	0.07	0.09	0.03
Pufa	0.02	0	0
Others	0.96	0.11	0.04
Pufa+Other	4.609	0.288	0.432
Total	100.00	100.06	100.00
SAT	25.24	26.24	25.45
MUFA	20.54	18.38	19.41
PUFA	53.26	55.32	55.09
Total	99.04	99.95	99.96
n-6	24.37	54.11	19.16
n-3	24.26	0.92	35.50
VLC n-3	20.60	0	0
EPA+DHA[4]	17.14	0	0
PUFA/SAT	2.11	2.11	2.16
n-6/SAT	0.97	2.06	0.75
LA/SAT	0.92	2.06	0.75
n-6/n-3	1.00	58.59	0.54
LA/ALA	20.43	60.89	0.54

[1]Fatty acid values are reported in percent of dietary oil mixture. Percent values are reported as means.

[2] MO = Menhaden oil mixture, SO = Safflower oil mixture, FXO = Flax seed oil mixture.

[3] Unidentified PUFA on chromatogram.

[4] ALA/EPA+DHA in Flax vs. Menhaden Diets = 2.07.

Table 3. Dietary Treatment Groups – Lipid Study[1]

Diet[2]	fa/fa Rats (fa)	Lean Rats (ln)
Flaxseed Oil Mixture	8 faFXO	8 lnFXO
Menhaden Oil Mixture	8 faMO	8 lnMO
Safflower Oil Mixture	8 faSO	8 lnSO
Pair Weighed Group (Safflower Oil Mixture)	8 faSO(PW)	8 lnSO(PW)
TOTALS	**32**	**32**

[1] Abbreviations for the 8 treatment groups are shown in the body of the table. Each treatment group consisted of n=8 rats.

[2] Flaxseed oil mixture diet = FXO [6% (w/w) flaxseed oil + 1% safflower oil + 2% coconut oil + 1% canola oil], Menhaden oil mixture diet = MO [7% (w/w) menhaden oil + 3% safflower oil], Safflower oil mixture diet = SO [7% (w/w) safflower oil + 2% coconut oil + 1% canola oil].

Table 4. Diet Formulations[1]

Ingredient	Flaxseed Oil (FXO)	Menhaden Oil (MO)	Safflower Oil (SO)
Dextrose (cerelose)[2] (g)	3387.6	3387.6	3387.6
Egg white (g)	1275.0	1275.0	1275.0
Fibre (cellulose) (g)	300.0	300.0	300.0
Mineral mix (g) (zinc-free)	210.0	210.0	210.0
Potassium phosphate (g)	32.4	32.4	32.4
Vitamin mix (g)	60.0	60.0	60.0
Choline (g)	15.0	15.0	15.0
Biotin mix (g) (200 mg/kg mix)	60.0	60.0	60.0
Zinc premix (g) (5.775 g Zn carbonate/kg mix)	60.0	60.0	60.0
Tert-butylhydroquinone (g)	0.084	0.084	0.084
Flaxseed oil[3] (g)	360.0	0.0	0.0
Safflower oil[4] (g)	60.0	180.0	420.0
Menhaden oil[5] (g)	0.0	420.0	0.0
Canola oil[4] (g)	60.0	0.0	60.0
Coconut oil (g)	120.0	0.0	120.0

[1] Diet ingredients were purchased from Harlan Teklad (Madison, Wisconsin) except dextrose, flaxseed oil, safflower oil, menhaden oil and canola oil. The above diet formulations were used to make 6 kg of each diet.

[2] Dextrose was purchased from Moonshiners (Winnipeg, Manitoba).

[3] Flaxseed oil was purchased from (Omega Nutrition, Vancouver, British Columbia).

[4] Safflower and canola oils were purchased from Vita Health (Winnipeg, Manitoba).

[5] Menhaden Oil was donated by Omega Protein (Reedville, Virginia, USA)

IV. RESULTS

The results recorded in this section are based on observations collected from two independent studies: (1) effects of dietary oil mixture experimental treatments for 9 weeks (or Lipid Study) and (2) effects of dietary zinc experimental treatments for 9 weeks (or Zinc Study) on different parameters in fa/fa and lean Zucker rats. Parameters measured in the Lipid Study include final body weights, total feed intakes, body fat pad weights, final pancreas weights, femur weights and lengths and zinc concentrations. Concentrations of various serum parameters and results from the oral glucose tolerance tests are also included. For the Zinc Study, final body weights, total feed intakes, fat pad weights, pancreas and relative pancreas weights, femur weights and lengths, femur zinc concentration, and serum zinc, and glucose already have been reported and discussed in K. Petroulakis' Master thesis. For the purpose of reporting pancreas zinc concentrations that were analyzed and C-peptide/insulin ratios that were calculated by this author, pancreas and relative pancreas weights, and serum insulin concentrations will be reported again in this thesis. Zinc Study parameters measured and reported in this thesis include serum c-peptide, leptin, triglyceride, and free fatty acid concentrations. This thesis also includes both insulin and metallothionein immunohistochemical staining results for both Lipid and Zinc Studies.

LIPID STUDY

Observations from the Lipid Study can be divided into the effects of genotype and the effects of dietary oil mixture treatment. Genotype effects refer to significant differences due to genetic differences between fa/fa and lean Zucker rats. Effects of

dietary oil mixture treatment refer to significant differences due to the manipulation of the dietary oil mixture in the diet. The results section will report effects due to genotype, dietary oil mixture treatment, and the interaction of genotype and dietary oil mixture (genotype X dietary oil mixture). Data will be presented as means ± the standard error of the mean (SEM), and will be represented in tabular format. If needed, 3 sets of tables will be used

(1) Always used: Tables that present the main effects for genotype (fa = fa/fa and ln = lean), dietary oil mixture treatment (FXO = Flax seed oil mixture, MO = Menhaden oil mixture, SO = Safflower oil mixture), and the genotype X dietary oil mixture interaction, and the means ± SEM for the six experimental diets (faFXO, faMO, faSO, lnFXO, lnMO, lnSO).

(2) If needed: Tables that present the main effects for genotype and the means ± SEM for fa/fa and lean rats.

(3) If needed: Tables that present the main effects for dietary oil mixture treatment and the means ± SEM for the FXO, MO, and SO groups.

BODY WEIGHT

Table 5 contains the final body weights of fa/fa and lean Zucker rats after 9 weeks of dietary oil mixture intervention. There were differences in final body weight due to genotype after the 9-week dietary lipid intervention trial (*Table 5*). The fa/fa rats had higher body weights at 9 weeks of the study compared to lean rats (*Table 6*). Dietary oil mixture did not alter final body weights of fa/fa or lean Zucker rats. Because of this finding, PW data of both fa/fa and lean Zucker rats were excluded from other analyses.

TOTAL FEED INTAKE

Feed intake was recorded daily as feed was replaced daily. Total feed intake for the 9-week period is shown in Table 7. There were significant differences due to genotype for the total feed intake of fa/fa and lean rats (*Table 8*). Dietary oil mixture did not alter total feed intake in fa/fa or lean Zucker rats. At week 9 of dietary intervention, the daily feed intakes of the fa/fa Zucker rat varied between 23.6 and 27.4 grams, while the daily feed intakes of the lean rat varied between 19.6 and 20.5 grams.

BODY FAT

Genotype influenced the epididymal and perirenal fat pads of these animals (*Tables 9 and 10*). The fa/fa rats had heavier epididymal and perirenal fat pads with larger relative epididymal and perirenal weights (expressed as g/100 g body weight) compared to lean rats. Epididymal and perirenal fat pad weights of fa/fa rats were 2.6- and 17.3-fold, respectively, heavier than the same fat pads of the lean rats. Dietary oil mixture treatment did not alter the weight or relative weight of the epididymal and perirenal fat pads.

FINAL PANCREAS WEIGHTS, FEMUR WEIGHTS AND LENGTHS

Table 11 contains the final pancreas weights, relative pancreas weights, and femur weight and length of fa/fa and lean Zucker rats. There were no differences due to dietary oil mixture treatment in these parameters. However, there were differences due to genotype as lean rats had significantly higher pancreas and relative pancreas weights, and

femur weights and lengths compared to fa/fa rats (*Table 12*). There was also a significant genotype x dietary oil mixture interaction for both pancreas weight and relative pancreas weight. Pancreas weights and relative pancreas weights (expressed in g pancreas/100 g body weight) of lean rats were 1.1 and 1.8 times, respectively, heavier than the pancreas weights and relative pancreas weights of fa/fa rats, thus implying the fa/fa pancreata contributed less to the overall body weights of the animals. Within the fa/fa group, the faSO rats had the lowest pancreas and relative pancreas weight than fa/fa rats fed the MO and FXO diets.

ZINC CONCENTRATIONS - PANCREAS, FEMUR AND SERUM

No differences were observed between fa/fa and lean rats for pancreas zinc concentration, total pancreas and relative pancreas zinc contents (*Table 13*). Genotype effects were observed for femur zinc and serum zinc concentrations (*Tables 13 and 14*). The fa/fa rats had higher femur zinc and serum zinc concentrations than lean rats. Dietary oil mixture type did not alter pancreas and femur zinc concentrations, or total and relative pancreas zinc contents for both fa/fa and lean Zucker rats. However, dietary oil mixture type did alter serum zinc concentrations (*Table 15*). The fa/fa and lean rats fed the SO diet had higher serum zinc concentrations than rats fed the FXO and MO diets, with the MO group having the lowest serum zinc concentrations of all three groups.

ORAL GLUCOSE TOLERANCE TESTS – SERUM GLUCOSE CONCENTRATIONS

The results for serum glucose concentrations during the OGTT are shown in Tables 16-18. There were no differences between fa/fa and lean rats in fasting serum glucose concentrations, with the concentrations within or close to the normal fasting blood glucose range of 4 to 7 mM. Fasting serum glucose concentrations of fa/fa and lean rats ranged between 6.81 to 7.60 mM and 6.43 to 7.40 mM, respectively.

Genotype differences (*Tables 16 and 17*) were observed at the t=15 minutes as fa/fa rats had significantly higher serum glucose concentrations compared to lean rats. Serum glucose concentrations of fa/fa rats ranged between 12.1 and 14.4 mM, while concentrations in lean rats varied between 9.3 to 10.4 mM at t=15 minutes. Genotype differences (*Tables 16 and 17*) were also observed in serum glucose concentrations measured at t=30 minutes. Compared to lean rats, fa/fa had significantly higher serum glucose concentrations at t=30 minutes. Serum glucose concentrations of fa/fa and lean rats were between 11.6 and 14.2 mM and 10.0 and 12.4 mM, respectively. Results observed at t=15 and t=30 minutes may indicate that the fa/fa rats had a delayed or hindered ability to clear glucose from the bloodstream after ingesting the glucose solution compared to lean rats. At t=60 minutes, fa/fa rats were able to lower their serum glucose concentrations to a similar concentration measured in lean rats at this particular time point.

SERUM INSULIN, C-PEPTIDE, C-PEPTIDE/INSULIN RATIO, LEPTIN, TRIGLYCERIDES, AND FREE FATTY ACIDS

Table 18 contains serum insulin, C-peptide, C-peptide/insulin, leptin, triglyceride and free fatty acid concentrations for both fa/fa and lean Zucker rats after 9 weeks of experimental diets. Genotype had a significant main effect on the serum parameters measured. The fa/fa rats had higher fasting serum insulin (17.5-fold), C-peptide (10-fold), leptin (26.7-fold), triglycerides (8.1-fold), and free fatty acid (1.2-fold) concentrations compared to lean rats (*Table 19*). The C-peptide/insulin ratio was significantly lower in fa/fa rats compared to the lean rats.

Fasting serum insulin and free fatty acid concentrations were also influenced by dietary oil mixture treatment (*Table 20*). The rats fed the SO diet had lower serum insulin concentrations than rats fed the FXO diet. Rats fed the MO diet had serum insulin concentrations that were not different from the SO and FXO groups. Serum fatty acid concentrations were lower in the rats fed the MO diet compared to rats fed the FXO and SO diets. Dietary oil mixture treatment did not influence C-peptide, leptin and triglyceride concentrations in fa/fa or lean Zucker rats.

ZINC STUDY

Observations from the Zinc Study can be divided into the effects of genotype and the effects of dietary zinc treatment. As in the Lipid Study, genotype effects refer to differences due to genetic differences between fa/fa and lean Zucker rats. Effects of dietary zinc treatment refer to significant result differences due to the manipulation of the dietary zinc. The results section will report effects due to genotype, dietary zinc

treatment, and the interaction of genotype and dietary zinc (genotype X dietary zinc). Data will be presented as means ± SEM, and will be represented in tabular format. If needed, 3 sets of tables will be used

(1) Always used: Tables that present the main effects for genotype (fa = fa/fa and ln = lean), dietary zinc treatment [ZD = Zinc deficient (5 ppm zinc), ZC = Zinc control (30 ppm zinc), ZS = Zinc supplemented (150 ppm zinc)], and the genotype X dietary zinc interaction, and the means ± SEM for the six experimental diets (faZD, faZC, faZS, lnZD, lnZC, lnZS).

(2) If needed: Tables that present the main effects for genotype and the means ± SEM for fa/fa and lean rats.

(3) If needed: Tables that present the main effects for dietary zinc treatment and the means ± SEM for the ZD, ZC, and ZS groups.

As stated in K. Petroulakis' thesis, body weights of fa/fa or lean rats fed ZD, ZC or ZS diets were not different. As a result, both fa/fa and lean PW groups were not used in the following analyses of the Zinc Study.

SERUM INSULIN, C-PEPTIDE, C-PEPTIDE/INSULIN RATIO, LEPTIN, TRIGLYCERIDES, AND FREE FATTY ACIDS

Only genotype had a significant main on fasting serum insulin, C-peptide, C-peptide/insulin, triglycerides, and free fatty acid concentrations of lean and fa/fa Zucker rats (*Table 21*). The fa/fa rats had 7-fold, 1.7-fold, and 1.6-fold higher serum C-peptide, triglyceride, and free fatty acid concentrations, respectively, compared to lean rats (*Table*

22). Lean rats had higher C-peptide /insulin ratio compared to the fa/fa rats. No significant main effect due to dietary zinc treatment was observed in these parameters.

For serum leptin concentrations, there was a significant main effect of genotype and a genotype x dietary zinc interaction. Lean rats had lower serum leptin concentrations than the fa/fa rats. The faZS rats had lower serum leptin concentrations than the faZD and the faZC rats.

FINAL PANCREAS WEIGHT AND ZINC CONCENTRATION

Table 23 contains the results for pancreas weight and pancreas zinc for the Zinc Study. Genotype effects were seen in final pancreas weights and relative pancreas weights but not pancreas zinc concentration or total and relative pancreas zinc contents. As noted in the thesis by K. Petroulakis (2000), the fa/fa rats had significantly lower pancreas weight and relative pancreas weight compared to lean rats (*Table 24*). Dietary zinc treatment did not influence final and relative pancreas weights in lean and fa/fa Zucker rats.

Dietary zinc treatment altered pancreas zinc concentrations, and total and relative pancreas zinc contents (*Table 25*). Rats fed the ZD diet had a 1.4-fold lower pancreas zinc concentration compared to the rats fed the ZC and ZS diets. As well, ZD rats had lower total and relative pancreas zinc contents compared to ZC and ZS rats.

Interactions between genotype and dietary zinc effects also were observed for pancreas zinc concentration (*Table 23*). The fa/fa rats fed the ZS diet had the highest pancreas zinc concentrations compared to rats fed the ZD or the ZC diets. Lean rats fed

the ZD diet had the lowest pancreas zinc concentrations compared to lean rats fed the ZC and ZS diets.

IMMUNOHISTOCHEMISTRY

Figure 2 contains images of pancreatic tissue sections of fa/fa and lean Zucker rats at 10X and at 40X magnifications. The tissue sections were stained with hematoxylin to help visualize various cells and tissue structures of the pancreas. As observed in Figures 2A and 2B, most of the tissue section consists of acinar cells, which are important in the exocrine function of the pancreas. As seen under 40X magnification (*Figures 2C and 2D*), the acinar cells are pyramidal in shape with zymogen granules at the apex, and the nucleus (more intense staining) at the base of each cell. The nuclei are purplish-blue, while the cytoplasm of each cell is light pink. These pancreatic tissue sections were taken from fasted rats; therefore, demand for pancreatic juice (digestive enzymes) by the small intestine was minimal. As a result, zymogen granules are quite visible in the acinar cells. Acinar cells cluster into larger units, or acini, with each cell's apex facing inwards. Occasionally, small ducts, which permit acinar zymogen drainage, can be seen in tissue sections. The ducts seen in Figures 2A and 2C are distinguished by their light colour. Cytoplasm from ductular and centroacinar cells that form these ducts stain lightly, while their nuclei stain purplish blue. Nuclei of these cells are typically elongated in shape or round. These particular ducts have a circular shape.

Islets of Langerhans, as seen in Figures 2A, 2B and 2D, are "islands" surrounded by acinar cells in the pancreatic tissue sections. In addition to their lighter staining, the islets are demarcated by a thin layer of reticular fibres that serve as borders between islets

and acinar cells. Islet cell type cannot easily be distinguished with hematoxylin staining. Therefore, different stains or immunohistochemistry is used to aid in the identification of different islet cells.

Pancreatic tissue morphology for both fa/fa and lean Zucker rats are similar (*Figures 2A –D*). Upon general examination, the major differences between fa/fa and lean rat pancreatic tissue sections are that fa/fa rat sections have more islets and a higher frequency of large and very large islets. Islets are harder to find in lean pancreatic tissue since they are less numerous and commonly smaller than the large islets of fa/fa rats.

As stated in the ***Materials and Methods*** section, fa/fa and lean Zucker rat pancreatic tissues for both Lipid and Zinc Studies were stained for insulin and metallothionein using immunohistochemistry. Results were gathered using qualitative analyses. Pancreatic tissue sections of each fa/fa and lean rat were observed at 2.5 to 40X power.

INSULIN

Lipid Study

Insulin immunostaining in pancreatic tissue sections of fa/fa and lean Zucker rats from the Lipid Study, as well as the negative insulin control are shown in Figure 3. Insulin-stained tissue sections were observed using the 2.5X and the 10X objective lenses. As seen in Figure 3, pancreatic tissue sections from both fa/fa and lean Zucker rats had very intense insulin staining. Genotype, but not dietary oil mixture treatment, appeared to influence insulin staining patterns. Compared to lean rats, pancreatic tissue sections of fa/fa rats had more islets and had more large-sized islets. The fa/fa islets

commonly were round in shape, had combinations of well-defined and poorly defined borders with patchy and uniform insulin staining. Larger islets were typically surrounded by smaller islets and usually were seen to project into surrounding acinar cells. This was not observed in pancreatic tissue from lean rats

Other islet cell types were hard to differentiate from the β-cells in the fa/fa islets. This is in contrast to islets observed in pancreatic tissue sections from lean rats where non-β-cells were easily distinguished from the β-cells in most islet sizes. In addition to fewer islets being observed in lean pancreatic tissue, less intense insulin immunostaining was observed in the larger islets of the lean rats.

Zinc Study

Insulin immunostaining in pancreatic tissue sections of fa/fa and lean Zucker rats from the Zinc Study, as well as the negative insulin control are shown in Figure 4. Again, genotype appeared to influence the observed staining patterns of pancreatic tissue sections of fa/fa and lean Zucker rats. Islets were more numerous and higher numbers of larger islets were observed in pancreatic tissue from fa/fa rats. Large islets of fa/fa rats were round, had well defined or poorly defined borders and usually had patchy insulin staining patterns. Commonly, large islets were surrounded by smaller islets and infiltrated (less-defined borders) into surrounding acinar cells.

Similar to the Lipid Study, non-β-cells were easily distinguished from β-cells in most islet sizes in lean compared to fa/fa pancreatic tissue. Although lean rats also had very intense insulin staining, the staining was not ubiquitous as that observed in fa/fa pancreatic tissue.

Dietary zinc treatment did not appear to influence the number, sizes, shapes and insulin staining patterns of the islets in pancreatic tissue from fa/fa or lean Zucker rats.

METALLOTHIONEIN

Lipid Study

MT immunostaining in pancreatic tissue sections of fa/fa and lean Zucker rats from the Lipid Study, as well as the positive and negative MT controls are shown in Figures 5 and 6. Pancreatic tissue stained for MT was observed using the 2.5X, 10X, and 40X objective lenses.

Genotype appeared to influence the MT immunostaining intensity (*Table 26*) in pancreatic tissue sections of fa/fa and lean Zucker rats. Intensity of MT immunostaining was based on a scale of one to four, where one represented no detectable MT staining and four represented easily discerned MT staining at 2.5X magnification. All pancreatic tissue sections of fa/fa rats had more intense MT staining, or were rated, as fours compared to lean rats. Lean pancreatic tissue had an average MT intensity of three, which meant MT staining was easily discerned at 10X magnification. Dietary oil mixture treatment did not appear to influence MT staining intensity in fa/fa pancreatic tissue; however, the dietary treatment appeared to influence MT staining intensity in lean pancreatic tissue. The lnFXO rats had less intense MT staining than lnMO and lnSO rats.

Genotype also appeared to influence MT immunolocalization in fa/fa and lean pancreatic tissue (*Table 27*). Detectable MT immunostaining was observed in the nucleus and cytoplasm of both acinar and islet cells of fa/fa pancreatic tissue. Positive MT immunostaining was commonly observed in peripheral islet cells. In contrast,

positive MT immunostaining was observed only in acinar nuclei and cytoplasm of lean pancreatic tissue.

Zinc Study

MT immunostaining in pancreatic tissue sections of fa/fa and lean Zucker rats from the Zinc Study, as well as the positive and negative MT controls are shown in Figures 7 and 8. Both genotype and dietary zinc treatment appeared to influence the MT immunostaining intensity observed in pancreatic tissue sections of fa/fa and lean Zucker rats (*Table 28*). The fa/fa rats had more intense MT immunostaining (average staining intensity of four) compared to lean rats (average staining intensity of two). The faZD rats had less intense MT immunostaining than the fa/fa rats fed the ZC and ZS diets. As well, lnZD rats had less intense MT immunostaining than the lnZC and ZS rats.

Genotype, and in the lean rats, dietary zinc treatment appeared to influence MT immunolocalization in pancreatic tissue sections (*Table 29*). Positive MT staining was observed in nuclei and cytoplasm of both acinar and peripheral islet cells of fa/fa rats. Except for the lnZC rats, MT staining of lean rats was observed only in acinar nuclei and cytoplasm. In the lnZC rats, positive MT immunostaining was also observed in the nucleus and cytoplasm of peripheral islet cells.

Table 5. Final Body Weights of fa/fa and Lean Zucker Rats Fed Dietary Oil Mixture Experimental Diets for 9 Weeks (Pair-weighed Data Included).[1,2]

	fa/fa				lean				F-Values[3]		
Parameter	faFXO	faMO	faSO	faPW	lnFXO	lnMO	lnSO	lnPW	Geno[4]	Lipid	Geno x Lipid
Final Body Weight (g)	615 ± 11[a]	606 ± 18[a]	616 ± 13[a]	574 ± 14[a]	385 ± 4[b]	399 ± 6[b]	394 ± 10[b]	366 ± 16[b]	<0.0001	NS	NS

[1] Values are means ± SEM for n=8 rats. Values with different superscript letters differ significantly by Duncan's multiple range test ($p<0.05$).

[2] fa = fa/fa Zucker rat, ln = lean Zucker rat, FXO = Flaxseed oil mixture diet, MO = Menhaden oil mixture diet, SO = Safflower oil mixture diet, PW = Pair-weighed (fed SO diet).

[3] F-values for main effects determined by two-way ANOVA.

[4] Geno = genotype, Lipid = dietary oil mixture treatment.

Table 6. Final Body Weights of fa/fa and Lean Zucker Rats Fed Dietary Oil Mixture Experimental Diets for 9 Weeks Based on Genotype (Pair-weighed Data Included).[1,2]

Parameter	fa/fa Rat	Lean Rat	F-Value
Final Body Weight (g)	603 ± 8	386 ± 5	< 0.0001

[1] Values are means ± SEM for n=32 rats.
[2] F values determined by ANOVA.

Table 7. Total Feed Intakes of fa/fa and Lean Zucker Rats Fed Dietary Oil Mixture Experimental Diets for 9 Weeks.[1,2]

	fa/fa			lean			F-Values[3]		
Parameter	**faFXO**	**faMO**	**faSO**	**lnFXO**	**lnMO**	**lnSO**	**Geno[4]**	**Lipid**	**Geno x Lipid**
Total Feed Intake (g)	**1786 ± 33[a]**	**1802 ± 42[a]**	**1781 ± 42[a]**	**1237 ± 20[b]**	**1286 ± 20[b]**	**1279 ± 27[b]**	**<0.0001**	**NS**	**NS**

[1] Values are means ± SEM for n=8 rats. Values with different superscript letters differ significantly by Duncan's multiple range test ($p<0.05$).

[2] fa = fa/fa Zucker rat, ln = lean Zucker rat, FXO = Flaxseed oil mixture diet, MO = Menhaden oil mixture diet, SO = Safflower oil mixture diet.

[3] F-values for main effects determined by two-way ANOVA.

[4] Geno = genotype, Lipid = dietary oil mixture treatment.

Table 8. Total Feed Intakes of fa/fa and Lean Zucker Rats Fed Dietary Oil Mixture Experimental Diets for 9 Weeks Based on Genotype.[1,2]

Parameter	fa/fa Rat	Lean Rat	F-Value
Total Feed Intake (g)	1790 ± 21	1267 ± 65	< 0.0001

[1] Values are means ± SEM for n=24 rats.
[2] F values determined by ANOVA.

Table 9. Epididymal and Perirenal Fat Pad Weights and Relative Weights of fa/fa and Lean Zucker Rats Fed Dietary Oil Mixture Experimental Diets for 9 Weeks.[1,2]

	fa/fa			lean			F-Values[3]		
Parameter	faFXO	faMO	faSO	lnFXO	lnMO	lnSO	Geno[4]	Lipid	Geno x Lipid
Epididymal Fat Pad Weight (g)	17.91 ± 0.56[a]	15.37 ± 1.10[a]	16.82 ± 1.16[a]	6.10 ± 0.36[b]	6.77 ± 0.58[b]	6.28 ± 0.39[b]	<0.0001	NS	NS
Relative Epididymal Fat (g/100g body wt)	2.99 ± 0.11[a]	2.61 ± 0.14[a]	2.78 ± 0.17[a]	1.62 ± 0.08[b]	1.74 ± 0.14[b]	1.63 ± 0.09[b]	<0.0001	NS	NS
Perirenal Fat Pad Weight (g)	23.58 ± 1.06[a]	22.17 ± 0.95[a]	24.12 ± 0.86[a]	7.13 ± 0.41[b]	7.16 ± 0.53[b]	7.36 ± 0.54[b]	<0.0001	NS	NS
Relative Perirenal Fat (g/100 g body wt)	3.92 ± 0.15[a]	3.79 ± 0.11[a]	4.00 ± 0.13[a]	1.90 ± 0.10[b]	1.84 ± 0.12[b]	1.90 ± 0.12[b]	<0.0001	NS	NS

[1] Values are means ± SEM for n=8 rats. Values with different superscript letters differ significantly by Duncan's multiple range test ($p<0.05$).

[2] fa = fa/fa Zucker rat, ln = lean Zucker rat, FXO = Flaxseed oil mixture diet, MO = Menhaden oil mixture diet, SO = Safflower oil mixture diet.

[3] F-values for main effects determined by two-way ANOVA.

[4] Geno = genotype, Lipid = dietary oil mixture treatment.

Table 10. Epididymal and Perirenal Fat Pad Weights and Relative Weights of fa/fa and Lean Zucker Rats Fed Dietary Oil Mixture Experimental Diets for 9 Weeks Based on Genotype.[1,2]

Parameter	fa/fa Rat	Lean Rat	F-Value
Epididymal Fat Pad Weight (g)	16.70 ± 0.58	6.38 ± 0.26	<0.0001
Relative Epididymal Fat (g/100 g body wt)	2.79 ± 0.08	1.66 ± 0.06	<0.0001
Perirenal Fat Pad Weight (g)	23.29 ± 0.59	1.35 ± 0.27	<0.0001
Relative Perirenal Fat (g/100 g body wt)	3.92 ± 0.37	1.88 ± 0.06	<0.0001

[1] Values are means ± SEM for n=24 rats.
[2] F values determined by ANOVA.

Table 11. Final Pancreas Weights, Relative Pancreas Weights, and Femur Weight and Length of fa/fa and Lean Zucker Rats Fed Dietary Oil Mixtures for 9 Weeks.[1,2]

	fa/fa			lean			F-Values[3]		
Parameter	faFXO	faMO	faSO	lnFXO	lnMO	lnSO	Geno[4]	Lipid	Geno x Lipid
Pancreas Weight (g)	1.53 ±0.10^{ab}	1.51 ±0.07^{ab}	1.27 ±0.08^{b}	1.53 ±0.07^{ab}	1.62 ±0.09^{a}	1.72 ±0.09^{a}	0.0111	NS	0.0306
Relative Pancreas Weight (g/ 100 g body weight)	0.26 ±0.02^{bc}	0.26 ±0.01^{b}	0.21 ±0.01^{c}	0.41 ±0.02^{a}	0.42 ±0.02^{a}	0.45 ±0.02^{a}	<0.0001	NS	0.0198
Femur Dry Weight (g)	0.532 ±0.005^{a}	0.542 ±0.013^{a}	0.531 ±0.014^{a}	0.565 ±0.010^{b}	0.602 ±0.008^{b}	0.591 ±0.017^{b}	<0.0001	NS	NS
Femur Length (cm)	3.22 ±0.02^{a}	3.29 ±0.06^{a}	3.23 ±0.02^{a}	3.51 ±0.01^{b}	3.55 ±0.03^{b}	3.52 ±0.02^{b}	<0.0001	NS	NS

[1] Values are means ± SEM for n=8 rats. Values with different superscript letters differ significantly by Duncan's multiple range test ($p<0.05$).

[2] fa = fa/fa Zucker rat, ln = lean Zucker rat, FXO = Flaxseed oil mixture diet, MO = Menhaden oil mixture diet, SO = Safflower oil mixture diet.

[3] F-values for main effects determined by two-way ANOVA.

[4] Geno = genotype, Lipid = dietary oil mixture treatment.

Table 12. Final Pancreas Weights, Relative Pancreas Weights, Femur Weight and Length of fa/fa and Lean Zucker Rats Fed Dietary Oil Mixture Experimental Diets for 9 Weeks Based on Genotype.[1,2]

Parameter	fa/fa Rat	Lean Rat	F-Value
Pancreas Weight (g)	1.44 ± 0.05	1.62 ± 0.05	0.0111
Relative Pancreas Weight (g/ 100 g body weight)	0.24 ± 0.01	0.42 ± 0.01	<0.0001
Femur Dry Weight (g)	0.535 ± 0.006	0.586 ± 0.007	<0.0001
Femur Length (cm)	3.25 ± 0.02	3.53 ± 0.01	<0.0001

[1] Values are means ± SEM for n=24 rats.
[2] F values determined by ANOVA.

Table 13. Pancreas Zinc Concentrations, Total Pancreas Zinc Content, Relative Pancreas Zinc Content, Femur and Serum Zinc Concentrations of fa/fa and Lean Zucker Rats Fed Dietary Oil Mixtures for 9 Weeks[1,2]

	fa/fa			lean			F-Values[3]		
Parameter	faFXO	faMO	faSO	lnFXO	lnMO	lnSO	Geno[4]	Lipid	Geno x Lipid
Pancreas Zinc (μg/g dry weight)	122.8 ±23.0	96.5 ± 8.3	122.5 ± 14.0	102.5 ± 4.4	84.7 ± 7.9	106.4 ± 6.5	NS	NS	NS
Total Pancreas Zinc Content (μg/pancreas)	55.0 ±10.4	45.1 ±2.9	46.2 ±4.4	43.5 ±3.4	45.0 ±4.9	55.9 ± 6.4	NS	NS	NS
Relative Pancreas Zinc (μg/100 mg wet weight)	3.69 ±0.77	2.98 ±0.15	3.70 ±0.43	2.83 ±0.12	2.76 ±0.25	3.18 ±0.25	NS	NS	NS
Femur Zinc (μg/g dry weight)	344 +5[a]	343 ±9[a]	359 ±6[a]	317 ±3[b]	304 ±8[b]	315 ±7[b]	<0.0001	NS	NS
Serum Zinc (μg/mL)	28.23 ±0.83[a]	25.01 ±1.34[a]	30.37 ±1.30[a]	19.69 ±0.81[b]	18.37 ±0.79[b]	21.62 ±0.48[b]	<0.0001	0.0005	NS

[1] Values are means ± SEM for n=8 rats. Values with different superscript letters differ significantly by Duncan's multiple range test ($p<0.05$).

[2] fa = fa/fa Zucker rat, ln = lean Zucker rat, FXO = Flaxseed oil mixture diet, MO = Menhaden oil mixture diet, SO = Safflower oil mixture diet.

[3] F-values for main effects determined by two-way ANOVA, [4] Geno = genotype, Lipid = dietary oil mixture treatment.

Table 14. Femur Zinc Concentrations and Serum Zinc Concentrations of fa/fa and Lean Zucker Rats Fed Dietary Oil Mixture Experimental Diets for 9 Weeks Based on Genotype.[1,2]

Parameter	fa/fa Rat	Lean Rat	F-Value
Femur Zinc (μg/g dry weight)	349 ± 4	312 ± 4	<0.0001
Serum Zinc (μg/mL)	27.87 ±0.80	19.82 ±0.49	<0.0001

[1] Values are means ± SEM for n=24 rats.
[2] F values determined by ANOVA.

Table 15. Serum Zinc Concentrations of fa/fa and Lean Zucker Rats Based on Dietary Oil Mixture Treatment.[1,2]

Parameter	FXO[3]	MO[3]	SO[3]	F-Value
Serum Zinc Concentration (μg/mL)	23.96 ±1.24[b]	21.69 ±1.14[a]	26.29 ±1.36[c]	0.0005

[1] Values are means ± SEM for n=16 rats for FXO and MO and n=16 for SO groups. Values with different superscript letters differ significantly by Duncan's multiple range test ($p<0.05$).
[2] F-values for main effects by two-way ANOVA.
[3] FXO = Flaxseed oil mixture diet, MO = Menhaden oil mixture diet, SO = Safflower oil mixture diet.

Table 16. Serum Glucose Concentrations During Oral Glucose Tolerance Tests of fa/fa and Lean Zucker Rats Fed Dietary Oil Mixture Experimental Diets for 8.5 Weeks.[1,2]

	fa/fa			lean			F-Values[3]		
Time (minutes)	faFXO	faMO	faSO	lnFXO	lnMO	lnSO	Geno[4]	Lipid	Geno x Lipid[4]
0 (Baseline)	7.2 ±0.3	6.8 ±0.4	7.6 ±0.6	6.9 ±0.2	6.4 ±0.6	7.4 ±0.5	NS	NS	NS
15	14.4 ±0.7[a]	12.1 ±1.1[a]	13.4 ±0.7[a]	9.3 ±0.3[b]	10.4 ±0.8[b]	9.8 ±0.7[b]	<0.0001	NS	NS
30	14.2 ±0.8[a]	11.6 ±1.4[a]	13.9 ±1.1[a]	10.0 ±0.7[b]	12.4 ±1.8[b]	11.1 ±0.6[b]	0.0147	NS	NS
60	10.9 ±0.7	9.2 ±0.8	9.8 ±0.5	9.5 ±0.8	9.4 ±1.2	10.5 ±1.0	NS	NS	NS

[1] fa/fa = fa/fa Zucker rat, ln = lean Zucker rat, FXO = Flaxseed oil mixture diet, MO = Menhaden oil mixture diet, SO = Safflower oil mixture diet.

[2] Values are for serum glucose concentration (mM) means ± SEM for n=8 rats. Values with different superscript letters differ significantly by Duncan's multiple range test ($p<0.05$).

[3] F-values for main effects determined by two-way ANOVA on log-transformed data. For repeated measures analyses, only genotype was significant. [4] Geno = genotype, Lipid = dietary oil mixture treatment.

Table 17. Serum Glucose Concentrations During Oral Glucose Tests of fa/fa and Lean Zucker Rats Fed Dietary Oil Mixture Experimental Diets for 8.5 Weeks Based on Genotype.[1,2]

Time	fa/fa Rat	Lean Rat	F-Value
15 minutes (mM)	13.30 ± 0.50	9.83 ± 0.83	<0.0001
30 minutes (log mM)	13.28 ± 0.65	11.12 ± 0.63	0.0147

[1] Values are means ± SEM for n=24 rats. There were no genotype differences at 0 and 60 minutes.
[2] F values determined by ANOVA, except log-transformed data for t=30 minutes.

Table 18. Fasting Serum Insulin, C-peptide, Leptin, Triglyceride, and Free Fatty Acid Concentrations of fa/fa and Lean Zucker Rats Fed Dietary Oil Mixture Experimental Diets for 9 Weeks.[1,2]

	fa/fa			lean			F-Values[3]		
Parameter	faFXO	faMO	faSO	lnFXO	lnMO	lnSO	Geno[4]	Lipid	Geno x Lipid
Insulin (ng/mL)	28.80 ±4.96[a]	28.42 ±4.49[a]	19.35 ±3.03[a]	2.02 ± 0.45[b]	1.39 ± 0.33[b]	0.97 ±0.09[b]	<0.0001	0.0196	NS
C-peptide (pM)	7736 ±530[a]	6776 ±652[a]	6342 ±546[a]	777 ±86[b]	757 ±152[b]	551 ±55[b]	<0.0001	NS	NS
C-peptide/ Insulin[5]	1.80 ±0.17[a]	1.56 ±0.15[a]	2.09 ±0.13[a]	2.78 ±0.33[b]	3.53 ±0.40[b]	3.40 ±0.19[b]	<0.0001	NS	NS
Leptin (ng/mL)	145.13 ± 8.12[a]	129.64 ± 6.48[a]	136.20 ± 3.37[a]	5.06 ± 0.81[b]	6.23 ± 1.25[b]	4.13 ± 0.53[b]	<0.0001	NS	NS
Triglycerides (mM)	16.99 ±2.29[a]	18.53 ±2.70[a]	14.21 ±1.91[a]	2.12 ±0.19[b]	1.82 ±0.35[b]	2.22 ±0.23[b]	<0.0001	NS	NS
Free Fatty Acids (mM)	0.39 ±0.04[a]	0.24 ±0.03[b]	0.34 ±0.02[a]	0.30 ±0.03[a]	0.22 ±0.01[b]	0.31 ±0.02[a]	0.0439	0.0001	NS

[1] Values are means ± SEM for n=8 rats. Values with different superscript letters differ significantly by Duncan's multiple range test ($p<0.05$).

[2] fa = fa/fa Zucker rat, ln = lean Zucker rat, FXO = Flaxseed oil mixture diet, MO = Menhaden oil mixture diet, SO = Safflower oil mixture diet.

[3] F-values for main effects determined by two-way ANOVA, except log-transformed data for insulin.

[4] Geno = genotype, Lipid = dietary oil mixture treatment

[5] C-peptide (pM)/insulin (pM).

Table 19. Fasting Serum Insulin, C-peptide, Leptin, Triglyceride, and Free Fatty Acid Concentrations of fa/fa and Lean Zucker Rats Fed Dietary Oil Mixtures for 9 Weeks Based on Genotype.[1,2]

Parameter	fa/fa Rat	Lean Rat	F-Value
Insulin (ng/mL)	25.51 ± 2.51	1.46 ± 0.20	< 0.0001
C-peptide (pM)	6951 ±341	695 ±62	<0.0001
C-peptide/ Insulin[3]	1.81 ±0.09	3.24 ±0.19	<0.0001
Leptin (ng/mL)	137.0 ± 3.7	5.14 ±0.53	<0.0001
Triglycerides (mM)	16.57 ±1.33	2.05 ±0.15	<0.0001
Free Fatty Acids (mM)	0.324 ±0.022	0.278 ±0.071	0.0439

[1] Values are means ± SEM for n=24 rats.
[2] F values determined by ANOVA.
[3] C-peptide (pM)/insulin (pM).

Table 20. Fasting Serum Insulin and Free Fatty Acid Concentrations of fa/fa and Lean Zucker Rats Based on Dietary Oil Mixture Treatment.[1,2]

Parameter	FXO[3]	MO[3]	SO[3]	F-Value
Insulin Concentration (ng/mL)	15.41 ±4.21[a]	14.91 ±4.11[ab]	10.16 ±2.79[b]	0.0196
Free Fatty Acid Concentration (mM)	0.348 ±0.026[a]	0.228 ±0.017[b]	0.327 ±0.015[a]	0.0001

[1] Values are means ± SEM for n=16 rats. Values with different superscript letters differ significantly by Duncan's multiple range test ($p<0.05$).
[2] F-values for main effects by two-way ANOVA; log-transformed data for insulin.
[3] FXO = Flaxseed oil mixture diet, MO = Menhaden oil mixture diet, SO = Safflower oil mixture diet.

Table 21. Fasting Serum Insulin, C-peptide, Leptin, Triglyceride, and Free Fatty Acid Concentrations of fa/fa and Lean Zucker Rats Fed Zinc Experimental Diets for 9 Weeks.[1,2]

	fa/fa			lean			F-Values[3]		
Parameter	faZD	faZC	faZS	lnZD	lnZC	lnZS	Geno[4]	Zinc	Geno x Zinc
Insulin (ng/mL)	47.4 ±6.1	29.3 ±5.9	36.2 ±8.8	1.7 ±0.3	1.7 ± 0.3	2.3 ±0.3	0.0001	NS	NS
C-peptide (pM)	7159 ± 725[a]	5198 ± 737[a]	6155 ± 618[a]	800 ± 182[b]	766 ± 120[b]	1067 ± 115[b]	0.0001	NS	NS
C-peptide/ Insulin[5]	0.93 ±0.05[a]	1.17 ±0.17[a]	1.22 ±0.18[a]	2.66 ±0.20[b]	2.84 ±0.36[b]	2.85 ±0.35[b]	<0.0001	NS	NS
Leptin (ng/mL)	165.53 ±12.20[a]	157.30 ±11.98[a]	126.37 ± 9.32[b]	5.22 ± 1.00[c]	4.40 ± 1.21[c]	6.56 ± 0.92[c]	<0.0001	NS	0.047
Triglycerides (mM)	30.29 ± 5.77[a]	33.86 ± 5.97[a]	41.00 ± 6.38[a]	16.00 ± 4.97[b]	19.57 ± 5.17[b]	26.71 ± 5.57[b]	0.0038	NS	NS
Free Fatty Acids (mM)	0.545 ± 0.075[a]	0.587 ± 0.052[a]	0.594 ± 0.066[a]	0.333 ± 0.040[b]	0.369 ± 0.046[b]	0.360 ± 0.062[b]	<0.0001	NS	NS

[1] Values are means ± SEM for n=7 rats. Values with different superscript letters differ significantly by Duncan's multiple range test ($p<0.05$).

[2] fa = fa/fa Zucker rat, ln = lean Zucker rat, ZD = Zinc-deficient diet, ZC = Zinc-control diet, ZS = Zinc-supplemented diet.

[3] F-values for main effects determined by two-way ANOVA, [4] Geno = genotype, [5] C-peptide (pM)/insulin (pM)

Table 22. Fasting Serum C-peptide, Leptin, Triglyceride, Free Fatty Acid Concentrations of fa/fa and Lean Zucker Rats Fed Zinc Experimental Diets for 9 Weeks Based on Genotype.[1,2]

Parameter	fa/fa Rat	Lean Rat	F-Value
C-peptide (pM)	6171 ± 421	878 ± 84	<0.0001
C-peptide/ Insulin[3]	1.10 ± 0.08	2.79 ± 0.14	<0.0001
Leptin (ng/mL)	149.73 ± 7.22	5.45 ± 0.61	<0.0001
Triglycerides (mM)	35.05 ± 3.46	20.76 3.04	0.0038
Free Fatty Acids (mM)	0.575 ± 0.036	0.354 ± 0.028	<0.0001

[1] Values are means ± SEM for n=21 rats.
[2] F values determined by ANOVA.
[3] C-peptide (pM)/insulin (pM)

Table 23. Pancreas Weights, Relative Pancreas Weights, Pancreas Zinc Concentrations, Total Pancreas Zinc Content, and Relative Pancreas Zinc Content of fa/fa and Lean Zucker Rats Fed Zinc Experimental Diets for 9 Weeks.[1,2]

	fa/fa			lean			F-Values[3]		
Parameter	faZD	faZC	faZS	lnZD	lnZC	lnZS	Geno[4]	Zinc	Geno x Zinc
Pancreas Weight (g)	1.21 ± 0.09[a]	1.26 ± 0.11[a]	1.40 ± 0.10[a]	1.57 ± 0.18[b]	1.63 ± 0.14[b]	1.73 ± 0.04[b]	0.0008	NS	NS
Relative Pancreas Weight (g/100 g body weight)	0.20 ± 0.02[a]	0.20 ± 0.02[a]	0.23 ± 0.01[a]	0.45 ± 0.05[b]	0.44 ± 0.03[b]	0.45 ± 0.01[b]	<0.0001	NS	NS
Pancreas Zinc (μg/g dry weight)	77.4 ± 3.3[bc]	87.5 ± 12.0[bc]	112 ± 6[a]	66.2 ± 3.0[c]	112 ± 6[a]	95.7 ± 10.7[ab]	NS	0.0001	0.0321
Total Pancreas Zinc Content (μg/pancreas)	29.1 ± 1.8[a]	38.3 ± 10.6[b]	47.9 ± 6.1[b]	31.0 ± 2.5[a]	49.5 ± 7.0[b]	53.0 ± 1.1[b]	NS	0.0007	NS
Relative Pancreas Zinc (μg/100 mg wet weight)	2.41 ± 0.11[a]	2.76 ± 0.45[b]	3.41 ± 0.32[b]	2.01 ± 0.08[a]	3.22 ± 0.17[b]	3.07 ± 0.11[b]	NS	0.0001	NS

[1] Values are means ± SEM for n=7 rats. Values with different superscript letters differ significantly by Duncan's multiple range test ($p<0.05$).

[2] fa = fa/fa Zucker rat, ln = lean Zucker rat, ZD = Zinc-deficient diet, ZC = Zinc control diet, ZS = Zinc-supplemented diet.

[3] F-values for main effects determined by two-way ANOVA, [4] Geno = genotype, Zinc = dietary zinc treatment

Table 24. Final Pancreas Weight and Relative Pancreas Weights of fa/fa and Lean Zucker Rats Fed Zinc Experimental Diets for 9 Weeks Based on Genotype.[1,2]

Parameter	fa/fa Rat	Lean Rat	F-Value
Pancreas Weight (g)	1.29 ± 0.06	1.64 ± 0.07	0.0008
Relative Pancreas Weight (g/ 100 g body weight)	0.209 ± 0.010	0.446 ± 0.020	<0.0001

[1] Values are means ± SEM for n=21 rats.
[2] F values determined by ANOVA.

Table 25. Pancreas Zinc Concentrations, Total Pancreas Zinc Content, and Relative Pancreas Zinc Content of fa/fa and Lean Zucker Rats Based on Dietary Zinc Treatment. [1,2]

Parameter	ZD[3]	ZC[3]	ZS[3]	F-Value
Pancreas Zinc Concentration (μg/g dry weight)	71.8 ±4.2[a]	99.6 ±4.1[b]	104.0 ±2.8[b]	0.0001
Total Pancreas Zinc Content (μg/pancreas)	30.0 ± 1.5[a]	43.9 ± 6.3[b]	50.4 ± 3.1[b]	0.0007
Relative Pancreas Zinc Content (μg/100 mg wet weight)	2.21 ± 0.09[a]	2.99 ± 0.24[b]	3.24 ± 0.17[b]	0.0001

[1] Values are means ± SEM for n=14 rats for all groups. Values with different superscript letters differ significantly by Duncan's multiple range test ($p<0.05$).
[2] F-values for main effects by two-way ANOVA.
[3] ZD = Zinc-deficient diet, ZC = Zinc control diet, ZS = Zinc-supplemented diet.

Table 26. Pancreatic Metallothionein Immunostaining Intensity in fa/fa and Lean Zucker Rats Fed Dietary Oil Mixture Experimental Diets for 9 Weeks[1,2].

Dietary Group	Pancreatic Metallothionein Staining Intensity[3]
faFXO	++++
faMO	++++
faSO	++++
lnFXO	++
lnMO	+++
lnSO	+++

[1] fa = fa/fa Zucker rat, ln = lean Zucker rat, FXO = Flaxseed oil-mixture diet, MO = Menhaden oil-mixture diet, SO = Safflower oil-mixture diet.
[2] Qualitative Analysis on n=8 for each group except n=7 for lnFXO.
[3] Metallothionein staining intensity: + = not detectable at 10X objective, ++ = barely visible at 10X objective, +++ = easily visible at 10X objective, ++++ = easily visible at 2.5X objective.

Table 27. Pancreatic Metallothionein Immunolocalization in fa/fa and Lean Zucker Rats Fed Dietary Oil Mixture Experimental Diets for 9 Weeks.[1,2]

Dietary Group	Pancreatic Metallothionein Staining			
	Acini		Islet Cells	
	Nucleus	Cytoplasm	Nucleus	Cytoplasm
faFXO	✓	✓	✓	✓
faMO	✓	✓	✓	✓
faSO	✓	✓	✓	✓
lnFXO	✓	✓		
lnMO	✓	✓		
lnSO	✓	✓		

[1] fa = fa/fa Zucker rat, ln = lean Zucker rat, FXO = Flaxseed oil-mixture diet, MO = Menhaden oil-mixture diet, SO = Safflower oil-mixture diet.
[2] Qualitative Analysis on n=8 for each group except n=6 for lnFXO, using 10X and 40X objective lenses.

Table 28. Pancreatic Metallothionein Immunostaining Intensity in fa/fa and Lean Zucker Rats Fed Zinc Experimental Diets for 9 Weeks[1,2].

Dietary Group	Pancreatic Metallothionein Staining Intensity[3]
faZD	+
faZC	++++
faZS	++++
lnZD	+
lnZC	++
lnZS	++

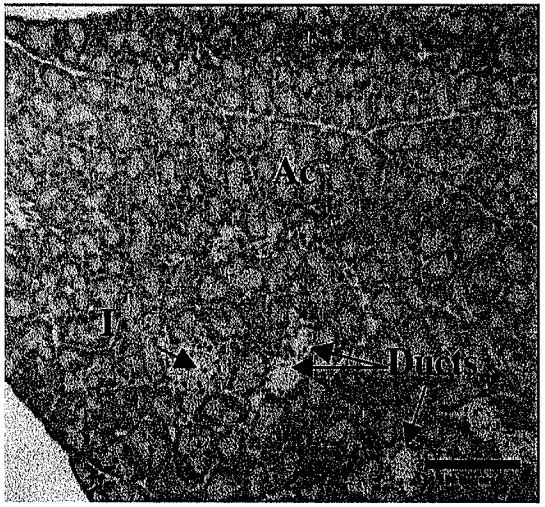
[1] fa = fa/fa Zucker rat, ln = lean Zucker rat, ZD = Zinc-deficient diet, ZC = Zinc control diet, ZS = Zinc-supplemented diet.
[2] Qualitative Analysis on n=7 for each group.
[3] Metallothionein staining intensity: + = not detective at 10X objective, ++ = barely visible at 10X objective, +++ = easily visible at 10X objective, ++++ = easily visible at 2.5X objective.

Table 29. Pancreatic Metallothionein Immunolocalization in fa/fa and Lean Zucker Rats Fed Zinc Experimental Diets for 9 Weeks.[1,2]

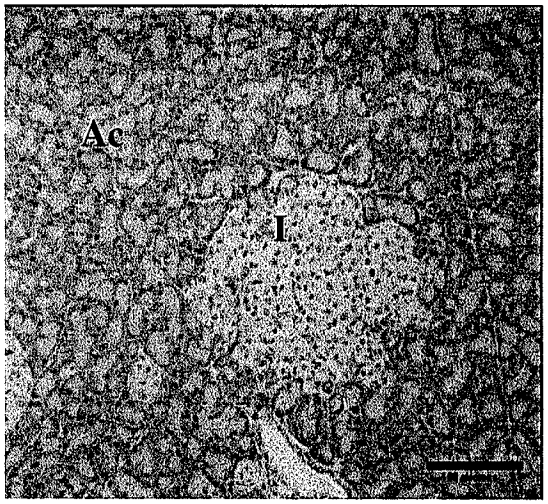
Dietary Group	Pancreatic Metallothionein Staining			
	Acini		Islet Cells	
	Nucleus	Cytoplasm	Nucleus	Cytoplasm
faZD	✓	✓	✓	✓
faZC	✓	✓	✓	✓
faZS	✓	✓	✓	✓
lnZD	✓	✓		
lnZC	✓	✓	✓	
lnZS	✓	✓		

[1] fa = fa/fa Zucker rat, ln = lean Zucker rat, ZD = Zinc-deficient diet, ZC = Zinc control diet, ZS = Zinc-supplemented diet.
[2] Qualitative Analysis on n=7 for each group, using 10X and 40X objective lenses.

Figure 2. Zucker Pancreatic Tissue: A. fa/fa and B. Lean Zucker Rat at 10X Magnification (Scale Bars = 100 μm), and C. fa/fa and D. Lean Zucker Rat at 40X Magnification (Scale Bars = 25 μm), I = islet, AC = Acinar Cells.



A.

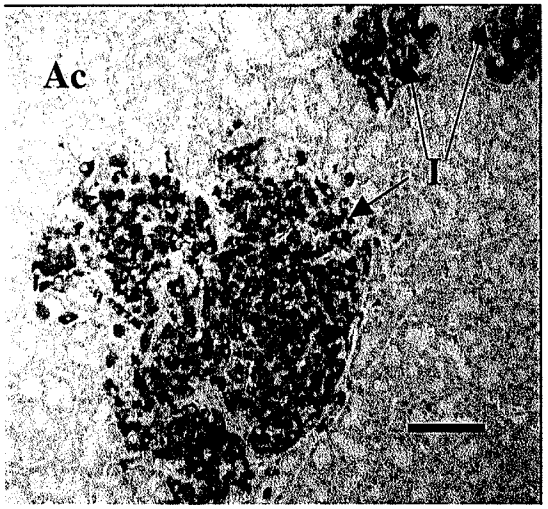


B.

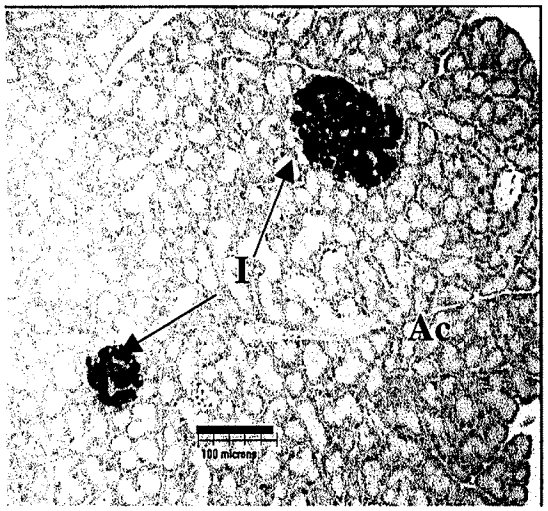
C.

D.

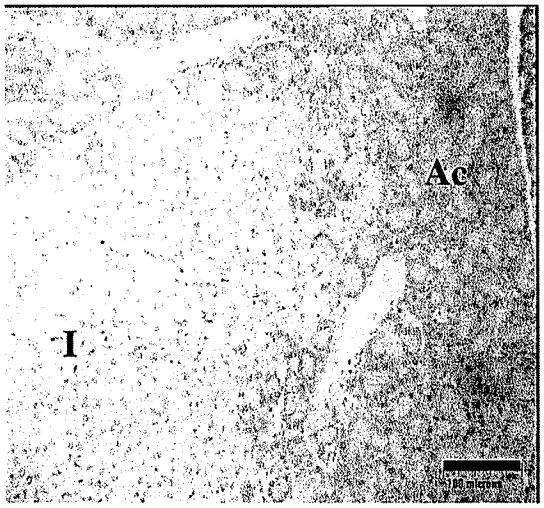
Figure 3. Immunohistological Staining for Insulin – Lipid Study. A. fa/fa and B. lean Zucker Rats, C. Negative Insulin Control (Scale Bars = 100 μm, I = Islet, Ac = Acinar Cells)



A.

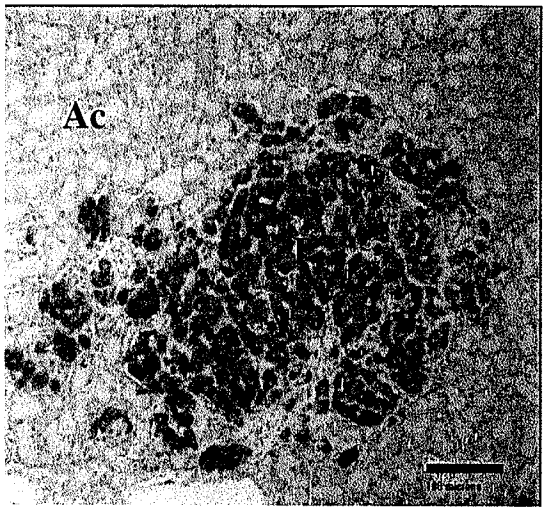


B.

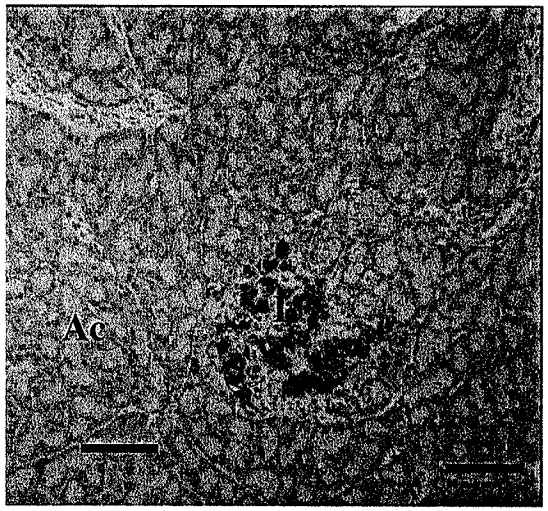


C.

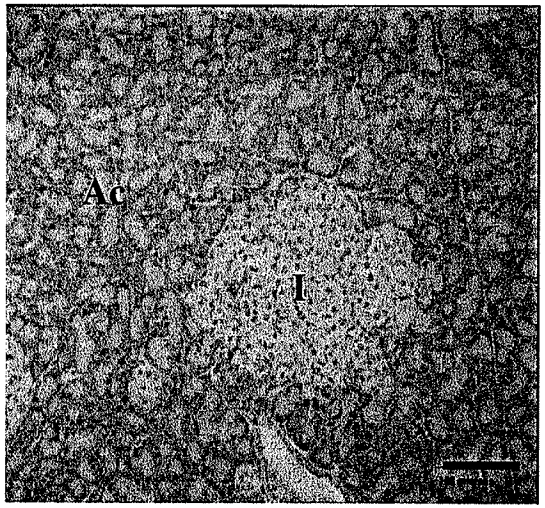
Figure 4. Immunohistological Staining for Insulin – Zinc Study: A. fa/fa and B. Lean Zucker Rats, and C. Negative Insulin Control (Scale bars = 100μm) (I = Islet, Ac = Acinar Cells).



A.

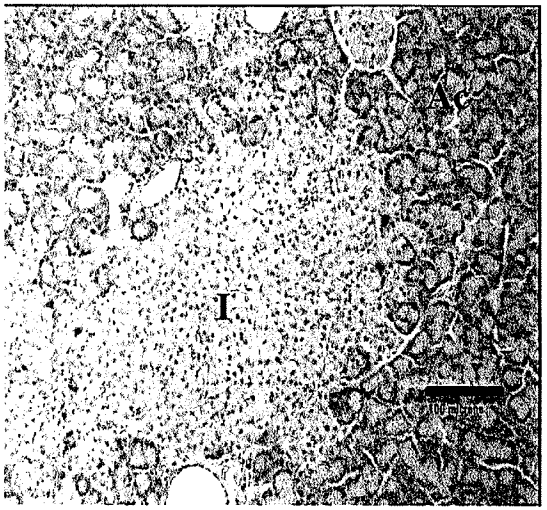


B.

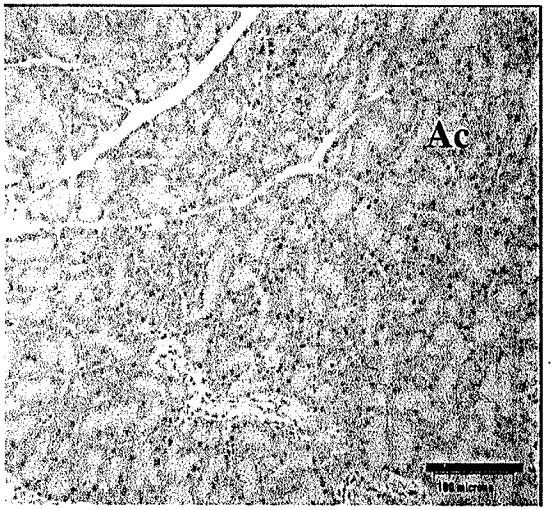


C.

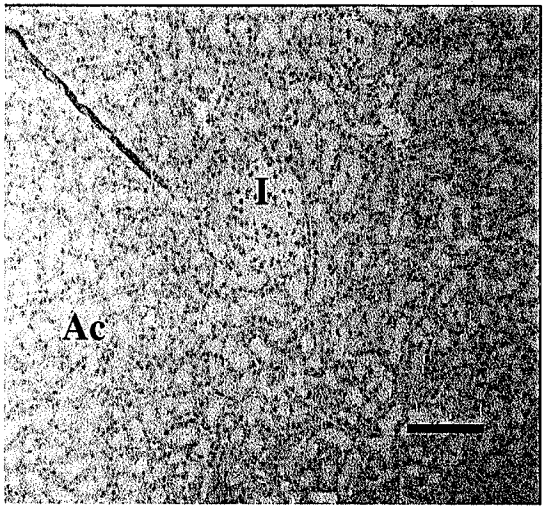
Figure 5. Immunohistological Staining for Pancreatic Metallothionein – Lipid Study: A. fa/fa and B. Lean Zucker Rats, C. Positive Control, and D. Negative Control (PBS buffer) (Scale bars = 100 μm) (I = Islet, Ac = Acinar Cells).



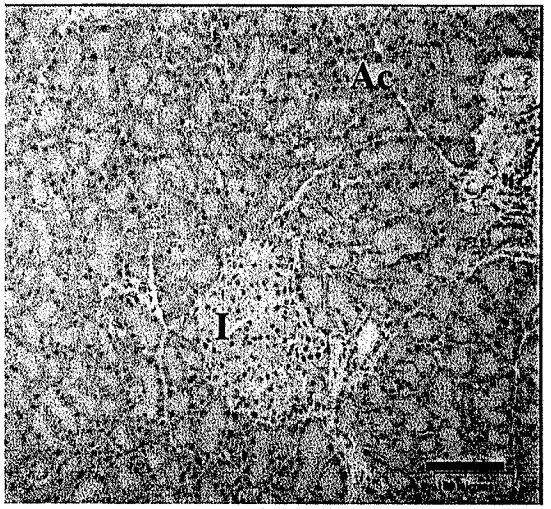
A.



B.

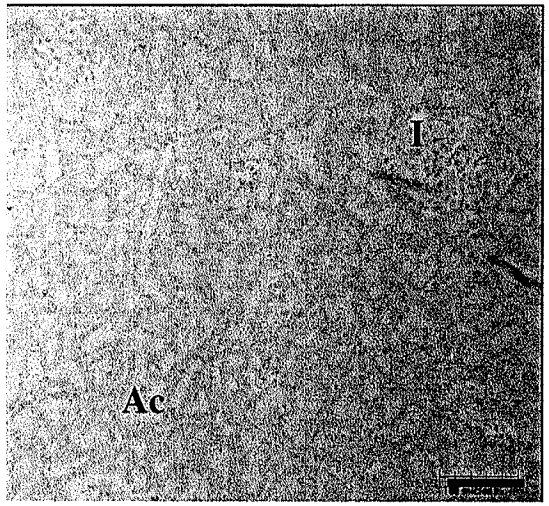


C.

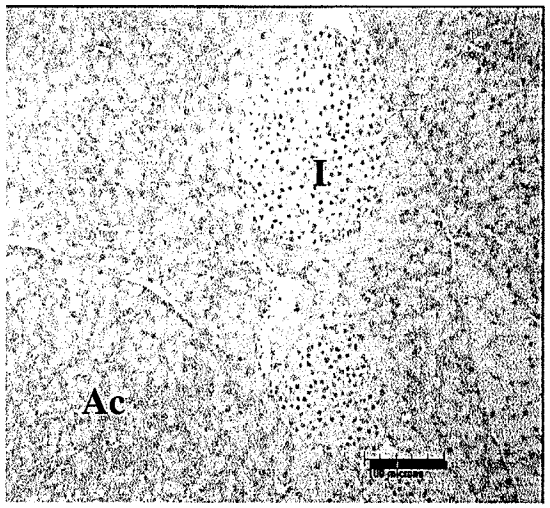


D.

Figure 6. Negative Controls for Immunohistological Staining for Pancreatic Metallothionein: A. IgG (Rat Tissue) and B. Metallothionein Null Mouse (Scale bars = 100 μm) (I = Islet, Ac = Acinar cells).

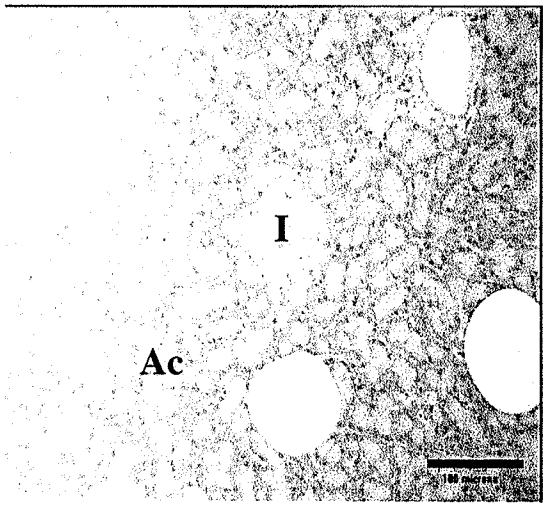


A.

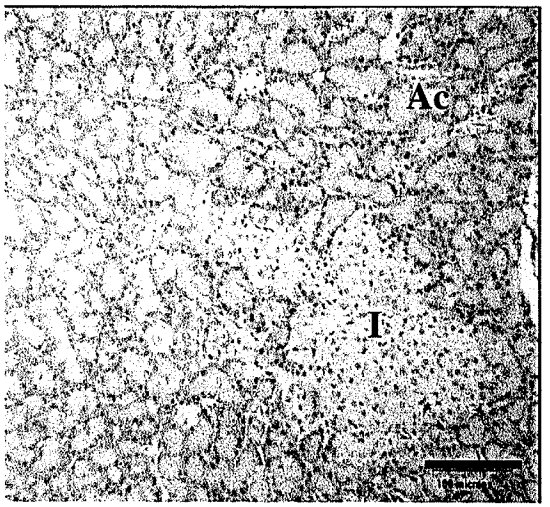


B.

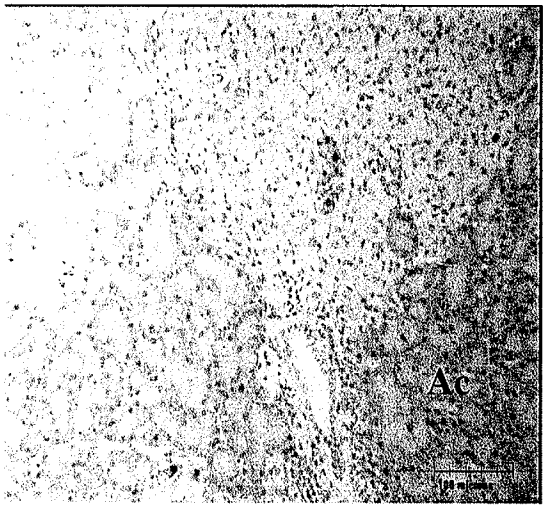
Figure 7. Immunological Staining for Metallothionein – Zinc Study: fa/fa Zucker Rat: A. faZD, B. faZC, C. faZS, D. Positive Control for Metallothionein (Scale Bars = 100 μm) (I = Islet, Ac = Acinar Cells, fa = fa/fa, ZD = Zinc-deficient, ZC = Zinc Control, ZS = Zinc Supplemented.



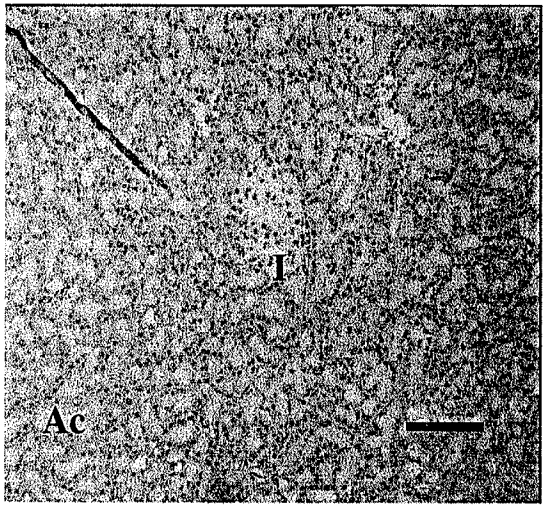
A.



B.

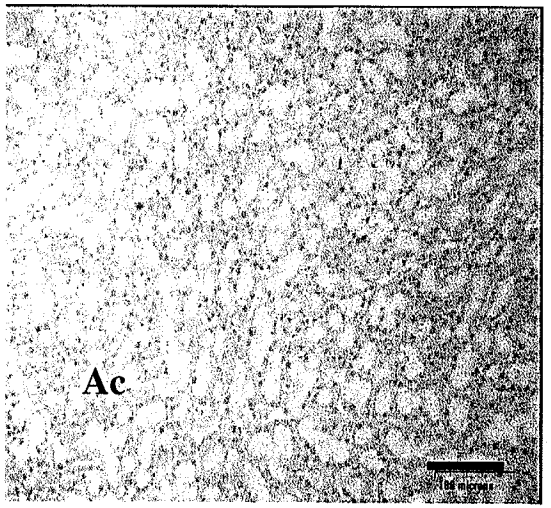


C.

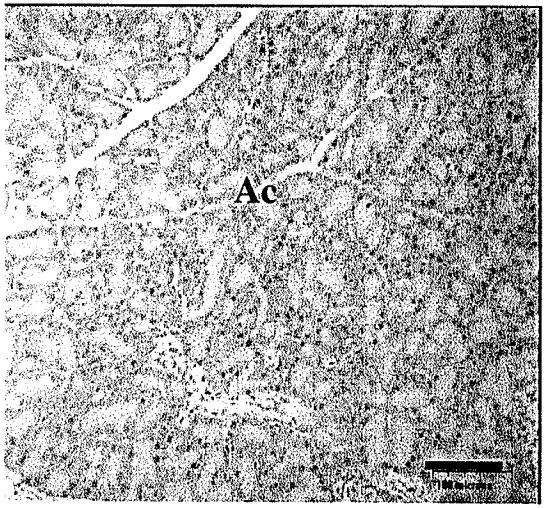


D.

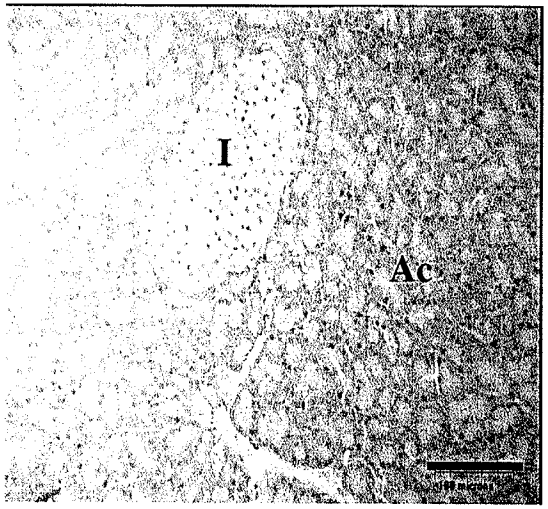
Figure 8. Immunohistological Staining for Pancreatic Metallothionein – Zinc Study - Lean Zucker rat: A. lnZD, B. lnZC, C. lnZS, and D. Positive Control for Metallothionein (Scale bars = 100 μm) [I = Islet, Ac = Acinar Cells, ln = Lean, ZD = Zinc Deficient (5 ppm}, ZC = Zinc Control (30 ppm), ZS = Zinc Supplemented (150 ppm)].



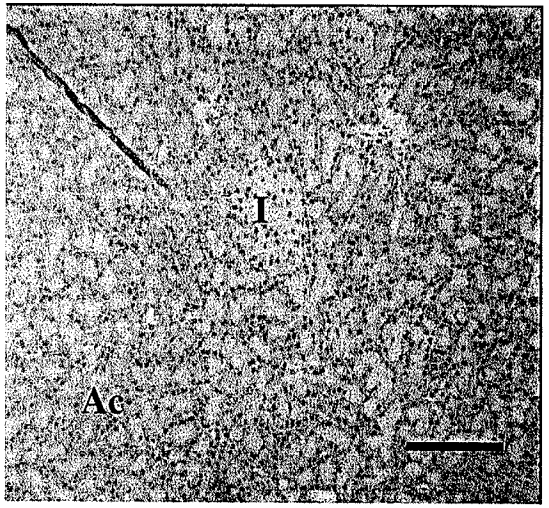
A.



B.



C.



D.

V. Discussion

LIPID STUDY

Body Weight

Body weight in this study only showed an effect due to genotype, as fa/fa rats were heavier than lean rats (*Tables 5 and 6*). There was no effect due to dietary oil mixture. Our result is supported by Liu et al. 2002, Petroulakis 2000, Cao et al. 1995, Ulmann et al. 1994, Guesnet et al. 1990, Stern et al. 1975, Zucker and Antoniades 1972, and Zucker and Zucker 1961. In addition to observing similar genotypic results, Mohan and associates (1991) also observed a dietary effect where fa/fa rats fed menhaden oil had lower body weights than fa/fa rats fed coconut or safflower oil. However, the researchers also observed lower total feed intakes in the menhaden oil group compared to coconut and safflower oil groups, which might explain the lower body weights in the menhaden oil. As well, the diets had different percentages of SAT, MUFA and PUFA that may have also contributed to the differences in body weights. A pair-weighed group would have controlled this confounding factor in this particular study and have made their results more viable.

There were no differences in body weight among the MO, FXO and SO groups, therefore, the PW group was not included in further analyses (*Table 5*). Thus, any changes due to dietary treatment were not confounded by differences in body weight.

Total Feed Intake

Total feed intake was higher in the fa/fa compared to the lean Zucker rats; no effects due to diet were observed (*Tables 7 and 8*). Similar genotype effects were observed (Liu et al. 2002, Petroulakis 2000, Mohan et al. 1991, Zucker and Antionades 1972, and Zucker and Zucker 1961), as hyperphagia is a characteristic of the fa/fa Zucker rat (Bray 1977). In addition, Mohan and associates (1991) observed that menhaden-oil-fed fa/fa rats had 27% and 18% lower total feed intake compared to coconut-oil–fed or safflower-oil-fed fa/fa rats, respectively.

Body Fat

Heavier weights and higher relative weights for epididymal and perirenal fat pads were observed in fa/fa compared to lean Zucker rats; however, no dietary effects on these parameters were observed (*Tables 9 and 10*). Petroulakis (2000) and Richards and colleagues (2000) also observed that genotype influenced epididymal and perirenal fat pad weights, while Johnson and colleagues (1971) also observed an effect of genotype on relative fat pad weights. As seen by Petroulakis (2000), perirenal fat pad weights were heavier than epididymal fat pad weights in fa/fa Zucker rats, thus contributing more to the total fat mass of these animals. In contrast, Richards and colleagues (2000) observed heavier epididymal than perirenal fat pad weights in fa/fa Zucker rats.

Varied results are published on the effect of oils at similar dietary levels on adipose depots in genetically lean rats, which can be compared to the lean Zucker rats of our study. In an 8-week study, where the dietary fat contributed to 26% of total calories (~12% w/w), Nagy and associates (1996) found lighter epididymal weights in male

Fischer rats fed DHA-rich diets compared to those fed safflower oil-rich diets. Although no fatty acid composition of each diet was reported, Nagy and colleagues used DHASCO (44.6% is DHA) for the DHA-rich diet. DHASCO contributed to 91% of the fat in the DHA-rich diet, or ~41% of fatty acids were DHA. The MO diet of our study contained 9.15% of the fatty acids as DHA, a much lower amount compared to the DHA-rich diet used by Nagy and colleagues. Therefore, it appears the DHA amount in the MO diet of our study was not high enough to influence epididymal fat pad weights in lean Zucker rats. However, Nagy and colleagues did not control SAT, MUFA and PUFA amounts in each respective diet; thus, it would be interesting if similar results would be observed with the same diet controls. Conversely, a future research consideration is controlling SAT, MUFA and PUFA amounts so individual fatty acids, such as DHA, could be tested.

Okuno and associates (1997) and van Amelsvoort and colleagues (1988) both fed moderate levels of dietary fat to genetically lean rats. Okuno and associates compared perilla oil (a source of ALA), while van Amelsvoort and colleagues compared linseed (flaxseed) oil effects on epididymal fat pad weights in male Sprague-Dawley and male Wistar rats, respectively. Van Amelsvoort and colleagues observed lower epididymal weights in rats fed diets that contained 14.4% (w/w) linseed oil for 4 weeks compared to those fed diets with similar amounts of sunflower oil, palm oil, olive oil, cocoa butter or coconut oil within the same amount of time. After a 12-week feeding trial, Okuno and associates reported lighter epididymal fat pad weights in rats fed 12% (w/w) perilla or safflower oil compared to those fed diets with similar amounts of beef tallow, or olive oil. Again, the limitation of both studies did not control SAT, MUFA and PUFA amounts, which may be influencing the results of their studies.

There are limited reports in the literature regarding the effects of dietary lipids on adipose depot weights of rodents with genetic obesity. Cleary and colleagues (1999) reported heavier retroperitoneal and epididymal fat pad weights in 11-12 week old male Zucker fa/fa rats fed 20% (w/w) coconut oil than those fed 20% (w/w) safflower oil. Using male ob/ob mice, Cunnane and associates (1986) found no difference in epididymal or relative fat pad weights in mice fed 10% (w/w) evening primrose oil (source of LA and γ-linolenic acid) or fed 10% (w/w) cod liver oil (source of EPA and DHA). Although both studies used different animal models, experimental designs and dietary fat amount, the studies give a hint on the effect of dietary fat on adipose tissue weight. It is possible that the lower amount of dietary fat (10% w/w) in Cunnane and associate's (1986) study was not high enough to influence adipose tissue weight. Cleary and colleagues used 20% (w/w) dietary fat and observed differences in adipose weight. As well, it is possible that using dietary fats with similar PUFA amounts may have similar effects on adipose weight in genetically obese animals. Cleary and colleagues (1999) compared safflower oil [high in PUFAs (LA), low in SATs] with coconut oil (high in SAT, low in PUFAs). Although Cunnane and associates (1986) provided no fatty acid analysis of the dietary oils used in the study, it is presumed there was a smaller difference in PUFA amounts between evening primrose oil and cod liver oil. It would have been interesting if Cunnane and associates also tested the oils at higher levels in the respective diets.

Similarly this idea could be used for our study. At 10% (w/w) dietary fat amount with controlled SAT, MUFA and PUFA amounts, long n-3 PUFAs did not influence fat pad weights. However, it would be interesting to determine if similar results would occur at higher levels of dietary, with similar controls in place.

In summary, examples available in the literature are limited because they are not just comparing the outstanding feature of the oil (e.g. flaxseed has high ALA), but they also are comparing unique fatty acid composition of that oil. Our study controlled SAT, MUFA and PUFA amounts to help us better determine the effect of long n-6 or n-3 PUFAs or very long n-3 PUFAs on obesity as indicated by epididymal and perirenal fat and relative pad weights. Our results indicate that at moderate dietary fat levels (10% w/w) with controlled SAT, MUFA and PUFA amounts, there is no difference in the effect of long n-6 or n-3 PUFAs or very long n-3 PUFAs on epididymal or perirenal fat or relative fat pad weights in fa/fa or lean Zucker rats.

Pancreas Weight

The observation (*Tables 11 and 12*) that lean rats had heavier pancreata and higher relative pancreas weights compared to fa/fa Zucker rats is supported by Petroulakis (2000) and Schneeman and colleagues (1983); however, the results are not supported by Meeris-Schwanke and associates (1998).

Two genotype X dietary oil mixture interactions were observed. Pancreata in lnFXO rats were lighter than the pancreata of lnSO and lnMO rats, while faMO rats had higher relative pancreas weights compared to faSO rats. Similar results for relative pancreas weight have not been observed or reported in the literature. Iritani and colleagues (1997) found no significant difference in pancreas weights of Wistar fatty and their lean littermates when fed diets of either 10% corn oil, 10% fish (cod liver) oil or 10% (9% beef tallow + 1% corn oil) beef tallow oil mixture. The Wistar fatty rat is the Wistar Kyoto strain with the *fa* gene of the Zucker fatty strain and is used as a model for

DM2 diabetes (Ikeda et al. 1997). Bégin and associates (1990) found no differences in pancreas weight in Sprague-Dawley rats (genetically lean) that were fed 5% (w/w) oil diets that had fish (fish source not stated), safflower, evening primrose (γ-linolenic acid) or a combination of these oils, and hydrogenated beef tallow.

To our knowledge, the effect of dietary oil, dietary oil mixtures, dietary oil mixtures with controlled SAT, MUFA and PUFA, or flaxseed oil on pancreas weights of lean and fa/fa Zucker rats have not been reported in the literature. A possible reason for our observation is the difference in fatty acid composition of the FXO diet compared to the MO and SO diets. ALA was a major component (35%) of the oil mixture of the FXO diet. This n-3 fatty acid is preferentially oxidized into energy instead of being elongated to EPA and DHA (Groff et al. 1995) that are more easily incorporated into membrane phospholipids or triglyceride storage. As a result, more fatty acids of the FXO diet are being metabolized by the body and less are being used for membrane phospholipid or triglyceride storage, and this may contribute to a smaller pancreas weight.

Given the potential differences in fatty acid metabolism and storage, it is possible that the MO diet may be affecting the body composition (adipose and lean tissue, body weight, and pancreas weight) of fa/fa rats. There was a *trend* for faMO rats to have lower body weights, lower fat pad weights (epididymal and perirenal), and higher pancreas weights compared to the faSO rats. However, there was a significant interaction between genotype and dietary oil mixtures when relative pancreas weight (pancreas weight / body weight) was calculated.

No fatty acid work was performed on the pancreas. It would be interesting to study the effects of the dietary oil mixtures on triglyceride concentration, as well as fatty

acid composition of phospholipid and triglyceride fractions of the pancreas in lean and fa/fa Zucker rats. As well, it would be interesting to examine the triglyceride concentrations in other lean tissues (e.g. liver, kidney, heart, gastrocnemius and soleus muscles). Triglyceride concentrations and fatty acid composition of tissues reflect fatty acid metabolism and they may also influence insulin sensitivity (Storlein et al. 1991, Hill et al. 1993, Luo et al. 1996, Fickova et al. 1998, and Giròn et al. 1999).

Femur Weight and Length

Genotype was the only main effect for femur weight and length as lean rats had heavier and longer femurs compared to fa/fa rats (*Tables 11 and 12*). These results are supported by Petroulakis (2000) who also found smaller femur weights and lengths in fa/fa Zucker rats.

Dietary oil mixtures did not significantly influence the femur weights and lengths of lean and fa/fa Zucker rats of this study. Femur weights and lengths were similar among the different dietary groups suggesting that at diets containing 10% (w/w) fat, both the fatty acid composition and the n-6/n-3 ratio do not influence these parameters in growing Zucker fa/fa and lean rats.

To our knowledge there are no studies that support or challenge these results. Claassen and associates (1995) found no differences in femur lengths or weights, but lower bone resorption and higher bone calcium in young male Sprague-Dawley rats fed test diet combinations of γ-linolenic (GLA) acid and EPA compared to rats fed a control diet for 6 weeks. All test and control diets had 8% (w/w) fat with test diets having varied low n-6/n-3 ratios (3:1, 1:1, and 1:3) and the control diet consisting of safflower and

linseed oil (n-6/n-3, 3:1). Both GLA and EPA by-pass the delta-6 desaturase enzyme, which is rate limiting to fatty acid elongation and desaturation and is believed to be susceptible to factors associated to osteoporosis (reviewed by Claassen et al. 1995). The SAT, MUFA and PUFAs of the diets were not controlled. It would be interesting to compare diets with controlled SAT, MUFA and PUFAs at higher dietary fat amounts, especially at fat amounts that are comparable to current estimated human fat intakes of industrialized countries of approximately 40% total calories from fat (Simopoulos 1999).

Serum Zinc, Femur Zinc, and Pancreas Zinc

Serum Zinc

Serum zinc concentrations indicate short-term zinc status. Serum zinc concentrations in the Lipid Study showed an effect due to genotype where the fa/fa rats had higher serum zinc concentrations compared to lean Zucker rats (*Tables 13 and 14*). This result is supported by Petroulakis (2000) where higher serum zinc concentrations were observed in fa/fa rats compared to lean Zucker rats.

As well, serum zinc concentrations were significantly influenced by dietary oil mixture (*Table 15*). Lean and fa/fa Zucker rats fed the SO diet, which contained linoleic acid (LA), had significantly higher serum zinc concentrations compared to rats fed the MO and FXO diets. To our knowledge, our study is the first to determine the effect of dietary oils or dietary oil mixtures on serum zinc concentrations in fa/fa Zucker rats.

It is possible LA acid may alter intestinal absorption of zinc. Although the mechanism of zinc absorption is not well understood, it is believed that zinc moves across the intestinal brush border as a free ion or as a ligand-zinc complex through

carrier-mediated transport (Song et al. 1988, Cousins and Hemp 1990, and Groff et al. 1995). Possible endogenous ligands of zinc include picolinic acid (tryptophan metabolite), glutathione, amino acids (histidine, cysteine, lysine and glycine), citric acid, prostaglandins and fatty acids (Groff et al. 1995, and Cousins and Hemp 1990).

Our finding suggests that LA may promote zinc absorption. This is in contrast to a study by Koo and Ramlet (1984) that found significantly lower serum zinc concentrations in adult Fischer-344 male rats fed a diet that contained LA for 6 weeks, thus indicating an effect of PUFAs on zinc metabolism. This study compared two diets that had 4%(w/w) dietary fat: one diet with LA and non-hydrogenated coconut oil (unsaturated diet, UNS) and the other diet with no LA and hydrogenated coconut oil (saturated diet, SAT). Each diet had similar amounts of saturated fatty acids (octadecanoic, decanoic, lauric, myristic and palmitic acids); however, the SAT diet had a much lower index of desaturation. The authors concluded that dietary PUFAs may influence zinc metabolism, possibly through "interfering with intestinal zinc absorption and/or the turnover of zinc" (Koo and Ramlet 1984).

In addition to differences in the animal models and age of the animals used (our study: 5-week-old Zucker fa/fa rat, Koo and Ramlet: adult (age not specified) Fischer-344 rats), there are two additional reasons could be contributing to the observed differences in serum zinc concentrations. First, Koo and Ramlet used 2 oil sources (non-hydrogenated coconut oil and LA) for the UNSAT diet, and LA was combined with fatty acids of carbon chains of 18 or less. Our SO diet, which was a mixture of safflower, coconut and canola oils, had a fatty acid composition that included LA, as well as a small amount of ALA (C18:3 n-3; 0.089%) and minimal amounts of fatty acids with 20 and 22

carbon chains. The oil combination in the SO diet possibly might have supported higher levels of zinc absorption, resulting in higher serum zinc concentrations. Second, which could be a more important reason, is that the SO diet contained a much higher LA (5.411% (w/w)) amount than the UNSAT diet (0.685% (w/w)) used in Koo and Ramlet's 1984 study. It is possible that small amounts of LA may inhibit, while larger amounts of LA may promote intestinal zinc absorption and/or turnover.

A similar pattern was found by Song and colleagues (1985) when they compared the effect of different doses of AA on zinc and prostaglandin metabolism (2-series: PGE_2, $PGF_{2\alpha}$ and 6-keto-$PGF_{1a\alpha}$) in 2-month-old Sprague-Dawley rats. The 2-series PGs are formed by the oxidation of AA catalyzed by cyclo-oxygenase. LA is the precursor of AA (*Figure 1*). The researchers found that a low amount (0.5 mg) of intraduodenally administered AA decreased the intestinal zinc transport rate of the rats. Rats received two doses of AA, at 24 and then at 4 hours before sacrifice and the rats also received one dose of radioactive zinc (10 μg ^{65}Zn) 1 hour before sacrifice. Interestingly, higher AA amounts (1.0 and 1.5 mg) increased intestinal zinc transport as observed in higher plasma ^{65}zinc content. Plasma $PGF_{2\alpha}$ significantly decreased in a dose-dependent manner, while $PGF_{2\alpha}$ and 6-keto-$PGF_{1a\alpha}$ were unchanged. However, all small intestinal PGs decreased in a dose-dependent manner with increasing AA dose. The researchers hypothesized that higher doses of AA may stimulate the transport of zinc in the small intestine by replacing mucosal PGE_2 but also stimulating PGE_2 biosynthesis in a rate-limiting manner for mobilization of the zinc-PGE_2-complex to the serosal side of the epithelial cells. The authors felt the inhibitory effect of low AA doses could replace PGE_2 in the mucosal side of the epithelial cells and make PGE_2 unavailable for zinc absorption and be too low to stimulate PGE_2 biosynthesis in these cells.

Different functions of PGs in intestinal zinc absorption have been determined in studies by Song and Adham (1978, 1979, and 1985) and Song and colleagues (1985). Song and Adham (1978 and 1979) indicated that PGE_2 enhanced zinc absorption and inhibited zinc excretion in both the small intestine and plasma, while the reverse was found for $PGF_{2\alpha}$. The researchers initially believed that PGE_2 was a zinc-binding ligand that chelated zinc on the mucosal side of the jejunum and enhanced zinc transport across the mucosal to the serosal side of the small intestine to where zinc would bind to plasma PGE_2 (Song and Adham 1979). Since then, research has identified that prostaglandins may act as ligands for zinc transport but may be more involved in zinc absorption by interacting with the cytosolic components of the epithelial cells of the small intestine through a possible transduction mechanism (Song et al. 1988).

Based on the above information, it is possible that the LA amount in the SO diet provided enough substrate for AA and later prostaglandin production that would enhance greater intestinal zinc absorption. LA is converted very efficiently to AA and both are readily incorporated into cellular membrane phospholipids (Linscheer and Vergroesen 1994).

To our knowledge no studies have looked at the effect of 3-series prostaglandins or n-3 fatty acids (ALA, EPA and DHA) on intestinal zinc absorption. As stated earlier, ALA competes with LA for the same elongation and desaturation enzymes. As well, EPA competes with AA for the cyclo-oxygenase enzyme and forms the 3-series PGs. The effects of n-3 fatty acids (EPA and DHA) on the intestinal absorption of other nutrients have been studied. For example, Thomson and colleagues (1990) found that dietary n-3 fatty acids (fish oil, EPA and DHA) reduced jejunal and ileal absorption of

glucose and some lipids (palmitic, stearic, oleic acids, LA, ALA and cholesterol) in diabetic and non-diabetic control rats. The anti-absorptive effect of dietary n-3 fatty acids could be extended to lowered intestinal zinc absorption since serum zinc concentrations of rats fed the MO and FXO diets were significantly lower than rats fed the SO diet.

Femur Zinc

Femur zinc concentration is an indicator of long-term zinc status. In this study, genotype was the only main effect for femur zinc concentrations as fa/fa rats had higher femur zinc concentrations (*Tables 13 and 14*). Petroulakis (2000) and Donaldson and colleagues (1987) support these results. There were no main effects due to diet. These results were expected as the level of dietary zinc was the same for each diet used in this study (30 ppm). Because, there were genotype effects for this parameter, the results suggest that the obese state of the fa/fa Zucker rats alters the zinc concentration of this organ.

Pancreas Zinc

Pancreas zinc, total zinc and relative zinc were not influenced by genotype or by diet (*Table 13*). Pancreas zinc concentration appeared to be unaffected by the obese state of the fa/fa Zucker rat model. This is in contrast to serum and femur zinc concentrations discussed earlier and other tissue, such as liver zinc concentrations measured in other studies. Petroulakis (2000) and Donaldson and colleagues (1987) measured lower liver zinc concentrations in fa/fa compared to lean Zucker rats. Although liver zinc concentrations were not measured in this study, it is possible that a similar pattern may be

observed since serum and femur zinc results agree with the other studies. It appears the pancreas zinc concentration is preserved in the obese state of this animal model.

Dietary zinc concentration was 30 ppm for each diet; however, each diet had different fatty acid compositions (*Table 2*). Therefore, pancreas zinc concentration also was preserved regardless of the fatty acid profiles of the diets in this study. Thus, pancreatic zinc metabolism appears to be similar for both the fa/fa and lean Zucker rats and is uninfluenced by the fatty acid composition of diets that contain 10% (w/w) dietary fat.

Using the fa/fa Zucker model, different patterns of zinc deposition emerge and are linked with obesity. In the Zucker fa/fa rat model, there is altered zinc metabolism, given the higher femur zinc storage and higher serum zinc in obese rats. However, pancreas zinc concentration was unaffected by obesity in diets with adequate dietary zinc and moderate levels of dietary fat.

Oral Glucose Tolerance Tests – Serum Glucose

OGTT is a "dynamic test for [pancreatic] β-cell function" (Gallwitz et al. 1998), we used the OGTT as one of the parameters to help assess the effect of the diets on the pancreatic function of the lean and fa/fa Zucker rats of our study. In addition, the OGTT is a common and the only clinical test that can be used to visualize and diagnose impaired glucose tolerance (Gallwitz et al. 1998), which is a characteristic of the fa/fa Zucker rat (Bray 1977, Ionescu et al. 1985, Pederson et al. 1991, Apweiler and Freund 1993, Berthiaume and Zinker 2002, and Liu et al. 2002).

The OGTT used in our study was performed on rats that were fasted for approximately 5 hours. Prior to the OGTT, fasting serum glucose concentrations were measured and there were no differences between lean and fa/fa Zucker rats (6.90±0.28 and 7.19±0.25 mmol/L, respectively). According to the literature, both lean and fa/fa Zucker rats are normoglycemic at a young age (6-7 weeks – Ionescu et al. 1985, and up to 9-10 weeks – Apweiler and Freund 1993). Later in their lifespan, the lean rats remain normoglycemic (Ionescu et al. 1985, Apweiler and Freund 1993, and Liu et al. 2002). Based on the Ionescu et al. (1985) and Apweiler and Freund (1993) studies, fa/fa Zucker rats develop mild hyperglycemia (basal glucose concentrations 7.4-7.6 mmol/L) at age 13-14 weeks and 12-13 weeks, respectively. However, the Liu et al. (2002) study did not observe mild hyperglycemia in the fa/fa Zucker rats. Instead, higher, yet normal fasting glucose concentrations were observed in 15-week-old fa/fa compared to lean Zucker rats (5.5 vs. ~4.2 mmol/L, respectively) (Liu et al. 2002). Techniques used in this thesis to minimize the stress of the rats, that is, the OGTT training, frequent handling of the rats, and blood collection via the sephanous vein, may explain the similar glucose concentrations observed in both the fa/fa and lean Zucker rats of our study. As well, rats in the above studies were fed laboratory chow that may have different diet compositions, influencing different dietary intakes that could also have affected the blood glucose concentrations of these animals.

The OGTT of our study indicated that the fa/fa Zucker rats had impaired glucose tolerance, as they had significantly higher serum glucose concentrations compared to the leans at both t = 15 and t =30 minutes (*Tables 16 and 17*). In other words, there was an effect of genotype at these two time points, which is supported in the literature (Ionescu

et al. 1985, Apweiler and Freund 1993, and Liu et al. 2002). Both Apweiler and Freund (1993) and Liu and colleagues (2002) observed higher plasma glucose concentrations in fa/fa compared to lean Zucker rats at all time points of the OGTT. However, Pederson and colleagues (1991) did not observe genotype differences in plasma glucose between 4 month (~ 16 week) old fa/fa and lean Zucker rats during OGTT. It should be noted the glucose load in the OGTT used by Pederson and colleagues was 1 g glucose/ kg body weight, whereas we used 0.7 g glucose/ kg body weight. In addition, 2 g glucose / kg body weight was used by both Apweiler and Freund (1993) and Liu and colleagues (2002). Thus, an oral glucose challenge between 0.7-2 g/kg is sufficient to detect differences in glucose tolerance between lean and fa/fa Zucker rats. It is possible that dietary effects may have been observed with a larger glucose dose during the OGTT.

No dietary effects were observed for fasting serum glucose or serum glucose concentrations during the OGTT. This observation implies that at moderate levels (10% w/w) of a dietary oil mixture with controlled SAT, MUFA, and PUFA amounts, glycemic control of the fa/fa Zucker rat is not influenced by the predominate type of PUFA (n-6 vs. long or very long n-3). To our knowledge, our study is the first to determine the effects of dietary oils or oil mixtures on the response to an oral glucose load in the fa/fa Zucker rat model.

Other dietary studies used different animal models, had different levels of dietary fat, and did not place similar controls on the fatty acid amounts of the dietary oils, thus, making it challenging to compare our observations to these studies. For example, Iritani and colleagues (1999) measured higher plasma glucose concentrations in Wistar fa/fa rats compared to their lean littermates during an OGTT. Wistar fa/fa rats are "obese,

hyperphagic, hyperinsulinemic, and have impaired glucose tolerance" (Irirtani et al. 1999), and eventually develop DM2 (Iritani et al. 1999). In the same study, Iritani and colleagues (1999) observed no differences in plasma glucose concentrations between Wistar fa/fa and lean rats receiving either corn oil (5 g/kg) intubation or no corn oil after the oral glucose load. Although it was an acute study that did not compare corn oil to other oils, the study by Iritani and colleagues (1999) also showed that the source of triacylglycerol (oil) did not influence the response of these rats to an oral glucose load.

In a long-term (25 weeks) dietary study, Minami and associates (2002) found no difference in glucose concentrations at any of the time points of an OGTT in male OLETF rats that were fed only chow, or chow supplemented with either 1 g/kg/d EPA or 1 g/kg/d high oleic safflower oil. OLETF, or Otsuka Long-Evans Tokushima Fatty rats are mildly obese and hyperinsulinemia, with most males (80-100%) developing hyperglycemia at 24 weeks of age (Mori et al. 1997 and Minami et al. 2002). Using the same rat model (OLETF), Sasagawa and colleagues (2001) found no difference in blood glucose concentrations at any of the time points of an OGTT of rats fed chow, or chow supplemented with either 2% lard, EPA, or AA for 17 weeks. However, Mori and associates (1997) observed lower plasma glucose concentrations at t=0, and t=120 minutes of an OGTT in male OLETF rats fed chow supplemented with 0.3 g/kg/d EPA-E (ester) compared to rats fed chow supplemented with 0.3 g/kg/d lard, olive oil, or safflower oil for 17 weeks.

After a 9-month feeding trial using male BHE/cdb rats, Bunce and colleagues (1992) found similar body weights within dietary fat levels (2 or 10% w/w) of menhaden oil, beef tallow or corn oil. Male BHE/cdb rats carry a genetic trait for DM2 that

manifests when the rat is 300 days old, starting with abnormal glucose tolerance (Bunce et al. 1992). However, the corn oil group had abnormal glucose tolerance (high glucose concentrations) when given an oral glucose dose (1 g /kg body weight), and they failed to return to pre-challenge glucose concentrations after 120 minutes..

As stated earlier, the OGTT is a dynamic test for pancreatic β-cell function. Serum glucose concentration, measured at the different time points of the OGTT, was used to assess pancreatic β-cell function in the fa/fa Zucker rat. However, it would have been beneficial to also measure both serum insulin and C-peptide concentrations to get a better picture of the effect of diet on dynamic pancreatic β-cell function, as both are secreted by the pancreatic β-cell. Overall, however, it appears that the benefits of PUFA interventions on glucose homeostasis may not be attributed to certain PUFA but rather to the PUFA/SAT ratio.

Serum Insulin, C-peptide and C-peptide/Insulin ratio

Serum Insulin

Genotype significantly influenced fasting serum insulin concentrations as fa/fa rats had higher serum insulin concentrations compared to lean Zucker rats (*Tables 18 and 19*). This result is supported in the literature (Liu et al. 2002, Petroulakis 2000, Mohan et al. 1991, and Pénicaud et al. 1987), as hyperinsulinemia is a characteristic of the insulin-resistant fa/fa Zucker rat (Bray 1977).

As well, dietary oil mixture was able to influence fasting serum insulin concentrations as both fa/fa and lean rats fed the SO diet had lower fasting serum insulin concentration compared to rats fed the MO and FXO diets (*Table 20*). Mohan and

associates (1991) also found fa/fa Zucker rats fed a 20% (w/w) safflower oil diet had the lowest fasting serum insulin concentrations compared to other fa/fa rats fed diets with 20% (w/w) menhaden or coconut oil. Similar dietary differences were not observed in lean Zucker rats. As stated earlier, differences in feed intakes and body weights in the Mohan et al. (1991) study could explain their fasting insulin results. Insulin resistance or the amount of insulin needed for glucose sensitivity can be decreased by reduced body weight, thus lowering serum insulin concentrations. It would seem that a higher fat amount in the diets used in the Mohan et al. (1991) study would encourage more differences in this parameter, as there was more LA and long chain n-3 PUFAs available in the safflower and menhaden oil diets, respectively. Another difference was that both fa/fa and lean Zucker rats were 10 weeks old at the beginning and 20 weeks old at the end of the Mohan et al. (1991) study compared to the rats used in our study (6 weeks at beginning and 15 weeks at end). Perhaps age is an important factor influencing how the lean rats respond to different dietary oils in this animal model.

Besides the Mohan et al. (1991) study, there is no additional literature that tested the effects of dietary oils on fasting serum or plasma insulin concentrations in fa/fa or lean Zucker rats. However, fasting insulin concentration was measured in other studies that tested the effects of different dietary oils in other rat subspecies. Using the male BHE/cdb rat, which is a genetic model of DM2, Bunce and colleagues (1992) did not observe differences in fasting insulin concentrations in rats fed beef tallow, corn or menhaden oil at dietary fat amounts of [2%(w/w) or 10% (w/w)]. Interestingly, Bunce and associates found higher fasting insulin concentrations only in rats fed 10% (w/w) compared to 2%(w/w) menhaden oil diets. They were unable to observe similar differences when they compared different dietary levels of corn oil or beef tallow.

Using male and female OLETF rats, another genetic model of DM2, Sasagawa and associates (2001) observed lower fasting serum insulin concentrations in rats fed diets with either 2% (w/w) EPA-rich oil or 2% (w/w) AA-rich oil compared to rats fed a standard chow diet. However, no differences in fasting insulin concentrations were observed between the EPA and AA groups. The EPA and AA oils used in the study were manufactured to contain high proportions of EPA and AA, respectively. Both studies (Bunce et al. 1992, and Sasagawa et al. 2001) gave interesting insights of dietary oil effects on animal models of DM2. However, as the fa/fa Zucker rat only has insulin resistance and impaired glucose tolerance, but does not develop DM2, there may be differences in glucose metabolism among the animal models. Therefore, comparing our results to the results of these studies may not be warranted.

No rat studies have compared the effects of dietary flaxseed oil (linseed oil) or other non-marine sources of n-3 fatty acids on fasting plasma or serum insulin concentrations. Using female C57BL/6J mice, Ikemoto and colleagues (1996) observed no differences in fasting plasma insulin concentrations when they fed the mice 60% calories from fat either as palm oil, lard, rapeseed (canola), soybean, safflower, fish oil, or perilla oil. C57BL/6J mice are genetically lean but are susceptible to developing obesity and insulin resistance if induced by diet (DIO). Perilla oil is a non-marine source of n-3 fatty acids as it contains ~ 60% ALA. To our knowledge, there are no rat or mouse studies that have tested the effect of flaxseed oil on fasting plasma or serum insulin concentrations.

There are variable results in studies that used lean rat models. Some studies found fish oil lowered (Bohov et al. 1997, Otto et al. 1991, and Baltzell et al. 1991),

elevated (Ezaki et al. 1992) or did not influence (Hill et al. 1993) fasting insulin concentrations. Bohov and associates (1997) also fed fish oil [10% (w/w)] to male hypertriglyceridemic rats and found no difference in their fasting insulin concentrations compared to those fed beef tallow. Variable results could be due to differences in animal models used: Sprague-Dawley rats were used by Baltzell et al., Otto et al., and Ezaki et al., while Wistar rats were used by Hill et al. and as part of Bohov et al.'s study. In addition, the variable results could be due to the level of fat used in the diets. Both Baltzell et al. (1991) and Otto et al. (1991) studies used 14.4% (w/w/) dietary fat, Ezaki et al. (1992) used 24% (w/w), Hill et al. (1993) used ~22% (w/w), while Bohov et al. (1997) used 10% (w/w/) dietary fat. Most studies compared different types of dietary oils; however, no study controlled the amounts of SAT, MUFA, and PUFA amounts to compare the effects of n-3 to n-6 fatty acid on fasting insulin concentrations in these animals.

In summary, there are no studies that tested the effect of fish oil on fasting serum or plasma insulin concentrations in fa/fa or lean Zucker rats. Few studies have tested the effects of fish oil on animal models of DM2, insulin resistance, or abnormal lipid metabolism. No studies have tested the effects of non-marine sources of n-3 fatty acids on rats, while perilla oil, a non-marine source of n-3 fatty acids [60% (w/w) ALA], was tested in C57BL/6J mice. To our knowledge, no studies have tested the effects of n-6, long n-3 and very long n-3 PUFAs on fasting insulin concentrations.

In our study, the higher fasting insulin concentrations in lean and fa/fa Zucker rats fed either flaxseed or fish oils imply greater insulin resistance, reduced hepatic insulin

clearance, or greater pancreatic β-cell function in these animals. However, this parameter should not be looked at in isolation as so many factors influence it.

Serum C-peptide and C-peptide/Insulin Ratio

Only genotype effects were observed for fasting serum C-peptide concentrations and for the C-peptide/insulin ratio (*Tables 18 and 19*). The fa/fa rats had higher serum C-peptide concentrations than the leans, whereas the leans had higher C-peptide/insulin ratios. Dietary oil mixture had no effect on these parameters. Fasting C-peptide concentration is a static clinical test for pancreatic β-cell function, which is commonly used to distinguish DM2 from type 1 DM, for monitoring endocrine function in chronic pancreatitis and as a measure for secretory action of β-cells (Gallwitz et al. 1998). Although the animals of this study did not have type 1 DM or develop DM2 or chronic pancreatitis, we were interested in using C-peptide as a measure of the secretory action of pancreatic β-cells of lean and fa/fa Zucker rats of this study. As well, fasting C-peptide is a better indicator of pancreatic β-cell function than serum insulin because once insulin is secreted, the liver takes up a large amount of insulin via the hepatic portal vein before it enters arterial blood circulation (Gallwitz et al. 1998). C-peptide does not share the same fate as insulin, as all of the metabolite enters general circulation and is eliminated from the body by the kidneys (Gallwitz et al. 1998). Therefore, as long as the kidneys are functioning normally, C-peptide concentrations more accurately reflect pancreatic β-cell function than insulin. Both lean and fa/fa Zucker rats were approximately 14 weeks when the study was terminated. At this age fa/fa rats have albuminuria but renal function

is considered normal (Kasiske et al. 1987). Thus, the C-peptide results can be used to infer about the pancreatic β-cell function of the rats of this study.

The C-peptide/insulin ratio was also used because the fa/fa rats had much larger fasting concentrations of C-peptide and insulin than the lean Zucker rats. Therefore, the C-peptide/insulin ratio was used as an index or a standardized representation of pancreatic β-cell function. For example, the higher C-peptide concentrations of the fa/fa rats indicated that they had greater pancreatic β-cell function or greater secretory action than lean rats. However, when the C-peptide/insulin ratio is compared (higher in leans vs. fa/fa Zucker rats), it appears the lean rat pancreas β-cell function is more efficient than the fa/fa rat pancreas. Proportionally, there was more C-peptide for every unit of insulin secreted by the lean rat compared to the fa/fa rat.

There are no examples in the literature that either support or refute our findings for both C-peptide concentrations and the C-peptide/insulin ratio. To our knowledge, no attempts have been made to measure and compare C-peptide and the C-peptide/insulin ratio in this animal model. However several reasons can be inferred from these results. The lean rat liver may be more efficient than the fa/fa liver at clearing insulin before this hormone enters the systemic circulation. Another possible reason is fa/fa pancreatic β-cells secrete more insulin but the conversion to this hormone and C-peptide from the precursor, proinsulin, may not be as efficient compared to pancreatic β-cells of lean rats. A combination of less efficient proinsulin to insulin conversion and a sluggish insulin clearance by the liver of the fa/fa rat could also be used to explain the ratio. At the time of our study, no products were available on the market that specifically measured serum proinsulin concentrations in rats. Therefore, it would have been interesting to determine

fasting proinsulin concentrations to get a better picture about pancreatic β-cell function after fasting in fa/fa and lean Zucker rats.

In summary, both higher fasting serum insulin and C-peptide concentrations in fa/fa compared to lean rats gave indirect indications of insulin resistance in fa/fa rats. Although higher fasting C-peptide concentrations in the fa/fa rat indicate greater pancreatic β-cell secretory action, the higher C-peptide/insulin ratio in the lean rat indicates more efficient conversion of proinsulin to insulin and possibly more efficient pancreatic β-cell and liver function. Dietary oil mixture had no effect on either improving or worsening pancreatic β-cell secretory action.

Serum Leptin

An effect of genotype was observed in serum leptin concentrations, as fa/fa had higher serum leptin concentrations compared to lean Zucker rats (*Tables 18 and 19*). Our result is supported in the literature where fa/fa Zucker rats had higher fasting (Liu et al. 2002) or basal (Vilà et al. 1998) serum or plasma leptin concentrations compared to lean Zucker rats. Hyperleptinemia is a characteristic of the fa/fa Zucker rat (Vilà et al. 1998).

Dietary oil treatments did not significantly influence serum leptin concentrations. As leptin expression (OB gene expression) occurs in adipose tissue, primarily through abdominal adipose depots, such as epididymal and peritoneal adipose (Ogawa et al. 1995), a possible reason can be ascertained. As stated previously, dietary oil mixture did not influence the epididymal and perirenal weights and relative weights of fa/fa or lean Zucker rats of our study. Thus, the observed lack of dietary effect in adipose tissue may have also resulted in not affecting the OB-gene expression in these adipose depots.

Measurement of OB-mRNA using Northern Analyses would help to provide more definitive answers.

To our knowledge, our study is the first to study the effect of dietary oil mixtures on fasting serum leptin concentrations in the fa/fa Zucker rat. As well, there is limited literature on the effect of dietary oil on genetic animal models of obesity and or diabetes, as most studies induce obesity by feeding high fat diets (DIO) to genetically lean animals. In a 12-week feeding trial, Hun and colleagues (1999) tested the effect of diets with 10% (w/w) oils (perilla, soybean or lard) or similar oils mixed with fish oil (DHA-EPA fish oil) on male KK-A^{Y}/Ta Jct mice, a genetic model of obesity and DM2. Although there was no difference in food intake, body weights or food efficiency ratios, mice fed the fish oil mixtures of each dietary oil had lower plasma leptin concentrations compared to the dietary oils alone. No differences were observed among the mice fed the single dietary oil diets. The researchers also observed lower epididymal weights in mice fed the perilla-fish oil and soybean-fish oil diets (Hun et al. 1999), which may partly explain the observed leptin results.

Hun and colleagues' (1999) results are interesting considering the EPA and DHA amounts (0.297% and 0.540%, respectively) in the fish oil mixture diets were lower than what was in the MO diet of this study (0.799% EPA and 0.915% DHA). These differences provide another example of the difficulty of relating results in one animal model to another and this is especially important when trying to relate results found in animal models to humans.

Variable results on the effects of dietary oils on plasma or serum leptin are found in the limited number of examples found in the literature. Cha and Jones (1998) did not

observe a decrease in fasting plasma leptin concentrations in Sprague-Dawley rats fed 20% (w/w) fish oil and 20% (w/w) safflower oil diets ad libitum for 10 weeks compared to rats fed 20% (w/w) beef tallow diet. This result occurred despite a decrease in perirenal fat pad weight and cell size only in the fish oil fed animals. Steinberg and Dyck (2000) reported higher post-meal serum leptin concentrations in Sprague-Dawley rats fed high fat (60% calories or ~29% w/w) safflower oil compared to fish oil for 2 and 4 weeks. Sprague-Dawley rats fed high sucrose diets with 14.4% (w/w) MaxEPA had higher post-meal plasma leptin concentrations than rats fed high sucrose diets with 14.4% (w/w) mixture of vegetable and animal fats both at 3 and 6 weeks of the feeding trial (Peyron-Caso et al. 2002). Using male C57Bl/ 6J mice, Wang and colleagues (2002) measured higher post-meal plasma leptin concentrations in mice fed the high fat edible tallow diet (58% calories from fat, ~28% w/w), compared to the high fat safflower and fish oil diets.

As shown, present literature consists mostly of DIO studies, each with various experimental designs, diets and different animal models, thus making it challenging to compare our results. It would have been beneficial to measure post-absorptive serum leptin concentrations to determine if there were dietary effects in the non-fasting fa/fa Zucker rat. As stated earlier, it would have been interesting to use molecular techniques to further our understanding of possible dietary effects on transcription and translation of the OB-gene. Leptin is the final product of many steps in its synthesis and only measuring serum leptin does not give any indication of what may be occurring in these steps.

Serum Free Fatty Acids and Triglycerides

Compared to lean rats, fa/fa Zucker rats had higher fasting serum free fatty acids (FFA) (*Tables 18 and 19*). This result is supported by Guesnet and colleagues (1990), and Liu and associates (2002). The fa/fa Zucker rats also had higher serum triglyceride concentrations than the lean rats (*Tables 18 and 19*).

An effect of diet was also observed for fasting serum FFA (*Table 20*). Lean and fa/fa Zucker rats fed the MO diet had significantly lower serum FFA concentrations compared to rats fed the FXO and SO diets. However, dietary oil mixture did not significantly influence serum triglyceride (TG) concentrations.

Our serum FFA result is in contrast to Fickova and colleagues (1998) who found no effect of menhaden oil on fasting serum FFA in Wistar rats in a 1 week feeding trial. Jucker and associates (1999) found no effect of MaxEPA on basal plasma FFA concentrations in Wistar rats of a 7 week dietary trial. However, Baltzell and colleagues (1991) reported lower fasting plasma FFA concentrations in Sprague-Dawley rats fed menhaden oil compared to rats fed corn oil or lard for 2 weeks. As indicated, all studies used genetically lean rats (Sprague-Dawley - Baltzell et al. 1991 and Jucker et al. 1999; Wistar – Fickova et al. 1998), in contrast to our study, which used both genetically lean and obese rats. Therefore, the differences in rat model could introduce differences in fat metabolism and overall metabolic differences that are unique to each rat model.

In addition, dietary fat (% w/w) amounts, as well as fatty acid composition varied in each study, which also could have contributed to the different results. Baltzell and colleagues (1991), which had similar FFA results to our study, used 14.4% (w/w) fat in their diets. The menhaden oil diet consisted of 12.5% (w/w) menhaden oil and 1.9%

(w/w) corn oil. The amount of EPA plus DHA made up 2.94% of the menhaden oil diet, compared to 1.71% the MO diet in our study. The authors did not include the daily feed intake of the rats; however, it is possible the rats had a similar feed intake as the lean rats of our study consumed 20 grams daily. Using this intake estimate, the EPA and DHA amount in the Baltzell et al. (1991) study was 0.588 g, which was higher than the fa/fa (0.405-0.470 g/d) and the lean (0.336-0.351 g/d) Zucker rats of our study. As well, both lean and fa/fa rats in our study consumed less EPA and DHA than the daily EPA and DHA intakes of 1.07 g and 1.62 g in the studies of Fickova et al. (1998) and Jucker et al. (1999), respectively. Both studies also used high dietary fat amounts of approximately 29% (w/w) (Fickova et al. 1998) and 28.5% (w/w) (Jucker et al. 1999). Although the EPA and DHA amounts were higher in these studies, the proportions of EPA and DHA amounts were smaller than used in both Baltzell et al. (1991) study and our study. The proportions of EPA and DHA in the diets were 0.03 for Fickova et al. (1998), 0.06 for Jucker et al. (1999), 0.20 for Baltzell et al. (1991), and 0.17 for our study. Thus, proportionally both the Baltzell et al. (1991) study and our study had a larger EPA + DHA presence.

To reiterate, our study controlled total amounts of SAT, MUFA and PUFA in all diets. The FFA results imply that at 10% (w/w) dietary fat with controlled SAT, MUFA and PUFA, very long chain n-3 fatty acids can effectively lower fasting serum FFA concentrations in fa/fa and lean Zucker rats. It would be interesting to investigate whether similar observations would occur at higher dietary fat amounts with controlled SAT, MUFA and PUFA.

Few human studies looked at the effects of dietary oil on FFA. Human studies that used volunteers with DM2, hyperlipoproteinemia (HP) and hypertriglyceridemia did

not observe a dietary effect of fish oil on fasting serum or plasma FFA concentrations (Nestel et al. 1984, Singer et al. 1985, Kamada et al. 1986, Singer et al. 1990, and Annuzzi et al. 1991). However, volunteers that ate fish or took fish oil supplements had significantly lower serum FFA during oral glucose tolerance testing. In a study of HP and normal volunteers, HP volunteers that ate mackerel (5.0 g EPA + DHA/d) for two weeks had significantly lower serum FFA during OGTT compared to the volunteers that had linseed (38 g linolenic acid/d) or sunflower (45 g linoleic acid/d) (Singer et al. 1990). The researchers also observed lower serum TG concentrations in the HP volunteers that had the mackerel.

Variable results for fasting TG were found in animal and human studies. Fish oil diets lowered (Baltzell et al. 1991, and Fickova et al. 1998) or had no effect (Jucker et al. 1999) on fasting plasma or serum TG concentrations. In human studies, subjects that ingested fish oil had lower fasting TG concentrations (Nestel et al. 1984, Singer et al. 1985, Singer et al. 1990, Annuzzi et al. 1991). Kamada and colleagues (1986) found no significant difference in fasting plasma TG concentrations in diabetic and non-diabetic volunteers before and after the consumption of 2.7 g sardine oil /d (0.89 g EPA + DHA/d) for 8 weeks. As indicated, our study had much lower amounts of EPA + DHA in the diets than used in the other studies, which could explain why plasma TG concentrations were unaffected.

This reasoning may also explain our FFA and TG results. It is possible the amount of EPA + DHA used in our diets was enough to influence fasting serum FFA but not enough to affect fasting serum TG concentrations. Our results indicate a trend for lnMO rats to have the lowest fasting serum TG concentrations; however, there was a

trend for highest TG in faMO rats. As the fa/fa Zucker rat has an altered lipid metabolism compared to lean Zucker rats, higher EPA + DHA amounts may be required to have a significant effect on fasting serum TG concentrations in this genotype.

Although no dietary effects were observed in body weight, and fat and relative fat pad weight, these parameters can be used to help explain the observed dietary effects on serum FFA. There was a trend for the faMO to have a lower body weight, fat pad and relative fat pad weights compared to faFXO and faSO rats. However, a trend was observed for lean rats, as lnMO had higher body weight, and epididymal and relative epididymal fat pad weights compared to lnFXO and lnSO rats. During fasting, FFA in the blood stream can be derived from the hydrolysis of VLDL from the liver by lipoprotein lipase of endothelial, skeletal, and adipose tissue, or from the hydrolysis of primarily the TG stores in fat pads by hormone-sensitive lipase. It is possible the hormone-sensitive lipase was more sensitive to the basal insulin concentrations, thus resulting in less hydrolysis of the adipose TG stores to FFA. With the trend of lower fat pad weights, but higher fasting serum TG in the faMO rat, it is possible the lipoprotein activity may have been lower and not as efficient in TG hydrolysis and uptake from the bloodstream. It is well known that phospholipid bilayers of cell membranes (adipose, skeletal, erythrocyte) are influenced by the dietary fat (Pan and Storlein 1993). Changes in membrane phospholipids can influence activities such as membrane-bound enzymes (e.g. lipoprotein lipase) and hormone receptors. It would have been interesting to measure enzyme activities for lipoprotein lipase or hormone-sensitive lipase to determine if there was an effect of diet on the activities of these enzymes.

Summary

Since fasting serum glucose, triglycerides, C-peptide concentrations were unaffected, but fasting serum insulin and free fatty acids were influenced by dietary oil mixture, n-3 fatty acids with controlled SAT, MUFA, and PUFAs at a level of 10% (w/w) in the diet minimally influence insulin resistance in these animals. Perhaps if the amounts in the diet were higher a greater effect might have been observed.

On other hand, the n-3 PUFA did not have any detrimental effects on insulin sensitivity. For management of DM2, n-3 PUFA may have other cardioprotective functions in addition to their hypolipidemic effects.

ZINC STUDY

Pancreas Zinc

Along with femur, liver and serum zinc that were discussed in Petroulakis's (2000) thesis, pancreas zinc was used as another assessment of zinc status of these animals. However, in contrast to femur, liver and serum zinc concentrations, genotype did not influence pancreas zinc concentration, total pancreas zinc content or relative pancreas zinc in the fa/fa rat of our study (*Table 23*). To our knowledge, there are no examples in the literature that have measured pancreas zinc in this animal model. Donaldson and colleagues (1987) did not measure pancreatic zinc and copper concentrations in lean and fa/fa Zucker rats when they tried to elucidate the effects of obesity and diabetes on tissue concentrations of these two minerals. Serfass and colleagues (1988) measured zinc concentrations in liver, brain, kidney, and tibialis muscle, but not the pancreas, to determine the developmental changes in tissue zinc

concentrations of fa/fa and lean Zucker rats. Schneeman and associates (1983) did not measure pancreas zinc concentrations when studying exocrine pancreatic enzymes in lean and fa/fa Zucker rats.

Our finding for pancreas zinc concentration is in contrast to the literature that has used other obese animal models. Simon (1998) and Southon and colleagues (1988) observed significantly lower pancreas zinc concentrations in db/db mice compared to their lean controls. The db/db mouse is an animal model of obesity and DM2, but also shares genetic homology to the fa/fa rat as the db/db mouse displays similar leptin resistance. In studies using ob/ob mice that are leptin-deficient, ob/ob mice had lower pancreas zinc concentrations compared to lean mice (Bégin-Heick 1985 and Kennedy and Failla 1987). The different animal models, as well as comparing rats (in this study) to mice (in the other studies) could explain the differing results. The db/db and ob/ob mice eventually develop diabetes, whereas the fa/fa Zucker rat does not develop this condition. Differences could also be due to different experimental designs such as length of study, dietary zinc concentration and age of animals used in the experiments.

As observed in femur and serum zinc concentrations (Petroulakis 2000), an effect of dietary zinc was observed in pancreas zinc concentrations (lowest in fa/fa and lean rats fed ZD diet) (*Tables 23 and 25*). Similarly to femur and serum zinc concentrations of rats in this study (Petroulakis 2000), the level of zinc (150 ppm) in the ZS diet also was not sufficient to elevate pancreas zinc concentrations.

The literature has conflicting results on the effect of dietary zinc on pancreas zinc. Pancreas zinc concentrations (Williams and Mills 1970, Canton and Cremin 1990) and pancreas zinc content (Van Wouwe and Uijlenbroek 1994) were significantly lower in

rats fed zinc deficient diets compared to their controls. However, a zinc deficient diet did not affect pancreas zinc concentrations in db/db mice (Southon et al. 1988, Simon 1998) or Chinese Hamsters (Boquist and Lernmark 1969). Simon (1998) observed that dietary zinc supplementation (300 ppm Zn) restored db/db pancreatic zinc concentrations to lean, non-diabetic values. Again, differences in results could be due to the animal models used (mice and hamsters vs. rats), levels of zinc used in the diets and differences in experimental design.

A genotype x dietary zinc interaction for pancreas zinc concentration also was observed in this study (*Table 23*). The lnZD rats had lower pancreas zinc concentrations than lnZC and lnZS rats, while faZS rats had higher pancreas zinc concentration than faZD and faZC rats. However, an effect of genotype was observed in pancreas weight and relative pancreas weights (Petroulakis 2000) (*Tables 23 and 24*), where leans had heavier pancreata and higher relative pancreas weights compared to the fa/fa Zucker rats. Although no significant genotype x dietary zinc interaction was observed in the femurs, livers or serum in the same fa/fa and lean Zucker rats, Petroulakis (2000) noted faZD and faZS rats had similar serum zinc concentrations. This trend was not observed in the lean rats causing Petroulakis (2000) to suggest both an altered and abnormal zinc metabolism in the fa/fa rat.

The pancreas zinc results also support the idea of an altered zinc metabolism in the fa/fa rat that responds differently to the three levels of dietary zinc compared to the lean Zucker rats. For example, the level of dietary zinc (150 ppm) in the ZS diet was not high enough to elevate pancreas zinc concentrations in the lean rat but it was sufficient to elevate pancreas zinc concentrations in the fa/fa Zucker rat. Alternatively, the fa/fa rat

pancreata may be more resistant to marginal zinc deficiency than lean rat pancreata, which could be a possible reason why the level of dietary zinc (5 ppm) in the ZD diet of this study did not result in lower pancreas zinc concentrations in the fa/fa rat.

Lean rats had heavier pancreas weights than fa/fa rats (Petroulakis 2000, *Tables 23 and 24*), but there were no genotype differences in pancreas zinc. The results imply that the fa/fa Zucker rats may be more efficient at pancreas zinc storage (more pancreas zinc in smaller amount of pancreas). However, upon closer observation, there is a trend of an effect of dietary zinc for pancreata weight: ZD< ZC <ZS (*Table 23*). As well, there also is a trend for genotype x dietary zinc interaction for pancreata weight: faZD, faZC and faZS pancreas weights were smaller than lnZD, lnZC and lnZS pancreas weights (*Table 23*). Combining the pancreas weight results with relative pancreas zinc and pancreas zinc concentrations suggest an altered, but not a more efficient zinc metabolism in fa/fa vs. lean Zucker rats. In other words, the results do not suggest the fa/fa rat is more efficient in zinc storage.

Serum C-peptide, C-peptide/Insulin Ratio

A significant effect due to genotype was observed in serum C-peptide and C-peptide/insulin ratio (*Tables 21 and 22*). The fa/fa Zucker rats had higher serum C-peptide concentrations than lean rats. Lean rats had a higher C-peptide/insulin ratio than fa/fa Zucker rats (*Tables 20 and 21*). To our knowledge, there is no published study that has measured C-peptide concentrations in fa/fa or lean Zucker rats.

The fasting serum-C-peptide results closely match the fasting serum insulin results reported by Petroulakis (2000), where fa/fa rats had higher insulin concentrations

compared to lean rats. As well, dietary zinc did not influence fasting serum insulin concentrations (Petroulakis 2000). As stated earlier, C-peptide and insulin are cleavage products of proinsulin and are secreted by the pancreatic β-cells. Also mentioned, fasting C-peptide was used as a static measure of pancreatic β-cell secretory action. Higher concentrations of C-peptide and insulin indicate increased pancreatic β-cell secretory action and insulin resistance, characteristics of the fa/fa Zucker rats. As well, the levels of dietary zinc used in the study were unable to worsen or improve insulin sensitivity or pancreatic β-cell function. It would have been interesting to do an OGTT to have a dynamic indication of pancreatic β-cell function. Using an OGTT that measured serum glucose, C-peptide and insulin concentrations would have given a more complete picture on the effect of dietary zinc on pancreatic β-cell secretory action.

As mentioned, the C-peptide/insulin ratio is a standardized representation of pancreatic β-cell function. Similarly to the *Lipid Study*, the lean rat pancreas appeared to be more efficient than the fa/fa rat pancreas with more C-peptide for every unit of insulin secreted. The result implies that the lean rat pancreas was more efficient at converting proinsulin to insulin. Measuring serum proinsulin would aid in the interpretation of these results.

Serum Leptin

Although many studies have measured serum or plasma leptin in fa/fa Zucker rats (Maffei et al. 1995, Iida et al. 1996, Vilà et al. 1998, and Liu et al. 2002), our study is the first to test the effect of dietary zinc on serum leptin concentrations in these animals. As

well, our study is the first to measure the effect of zinc supplementation on serum leptin concentrations in a rat model of genetic obesity.

As confirmed in previous studies (Maffei et al. 1995, Iida et al. 1996, Vilà et al. 1998, and Liu et al. 2002), a significant effect of genotype was observed in serum leptin concentrations (*Tables21 and 22*). The fa/fa Zucker rats had significantly higher serum leptin concentrations compared to lean rats.

No significant effect due to dietary zinc was observed in serum leptin concentrations. However, a significant genotype x dietary zinc interaction in serum leptin concentrations was observed only in fa/fa Zucker rats. The fa/fa Zucker rats fed the ZS diet had significantly lower serum leptin concentrations than fa/fa rats fed the ZC and ZD diets.

Observing no effects of dietary zinc on serum leptin concentrations parallels the lack of dietary effects on body weight, fat pad and relative fat pad weights (perirenal, epididymal and total) (Petroulakis 2000). These results (Petroulakis 2000) are considered when looking at serum leptin concentrations in these rats, since leptin concentration is positively correlated with body fat in both rodents (rats and mice) and humans (Maffei et al. 1995, and Frederich et al. 1995). As well, Petroulakis (2000) reported no effect of dietary zinc on fasting serum insulin concentrations, which also may partially explain our observations for serum leptin. Both animal (rodent) and human studies show that chronically and acutely high insulin concentrations stimulate leptin gene expression, secretion and elevated plasma leptin concentrations (Saladin et al. 1995, Kolaczynski et al. 1996 and Saad et al. 1998). However, these parameters do not explain the genotype x dietary interaction observed in fa/fa rats.

Low leptin concentrations were observed in dietary zinc deficiency trials that involved lean rodents (rats or mice) and non-obese humans. Zinc-deficient rats have lower plasma leptin concentrations (Manigan et al. 1998), and reduced ob mRNA synthesis and leptin secretion in adipocytes compared to zinc sufficient ad libitum and pair-fed controls (Ott and Shay 2000). Gaetke and colleagues (2002) similarly found lower plasma leptin concentrations in zinc deficient rats compared to zinc sufficient (ad libitum) controls. However, they also found serum leptin concentrations of pair-fed rats did not differ significantly from either zinc deficient or zinc sufficient (ad libitum) rats, causing them to conclude food intake was responsible for low serum leptin concentrations in zinc deficient rats. In a human trial, Mantzoros and colleagues (1998) measured decreased serum leptin concentrations in young, healthy, non-obese men who were made marginally zinc deficient through a zinc-restricted diet. Body weights of the men were not significantly altered after the dietary zinc restriction.

The few dietary zinc or zinc supplementation studies available in the literature suggest that leptin concentrations are *elevated* in *in vivo* or *in vitro* systems supplemented with zinc. Chen and Lin (2000) observed that DIO C57BL/6J mice fed sucrose solutions supplemented with 20 mg/l zinc acetate (DIOZn) *elevated* serum leptin concentrations compared to the unsupplemented DIO controls. Both DIO groups had similar body weights and percent body fat, but they had higher serum leptin than the non-obese controls. Hyperglycemia and hyperinsulinemia commonly occur in this DIO mouse model. Chen and Lin (2000) found attenuation of these conditions to non-obese values in the DIO mice supplemented with zinc. A low dose of zinc (0.1 mM) augmented, while a pharmacological dose (1mM) of zinc attenuated leptin secretion in adipocytes from

C57BL/6J mice made diabetic with STZ (Chen et al. 2001). In an *in vivo* study using STZ-treated C57BL/6J mice that received 20 mg/l zinc acetate in their drinking water, serum leptin concentrations of these mice elevated to the concentrations of their non-diabetic controls (Chen et al. 2000).

An *in vitro* study using human adipocytes suggested significantly higher leptin production in adipocytes incubated in a solution that contained 0.2 mM zinc (Chen et al. 2000). The study used adipocytes from subcutaneous tissue of non-obese women and was conducted due to an earlier observation of consistently higher leptin and lower zinc plasma concentrations in obese men compared to non-obese men (Chen et al. 2000). Unfortunately, the authors did not test adipocytes from obese volunteers or use zinc doses lower than the pharmacological dose used for the *in vitro* study. As well, it would have been interesting to test the effect of dietary zinc supplementation on serum leptin concentrations in both obese and non-obese subjects. This would have given information of *in vivo* effects of zinc on leptin production and secretion.

Mantzoros and colleagues (1998) observed significantly increased serum leptin concentrations in marginally zinc-deficient men when they received zinc supplements that provided either 30 to 60 mg elemental zinc/day. Although this study is an example for zinc supplementation from marginal zinc deficiency, it does indicate the influence of zinc on leptin concentrations *in vivo*.

However, the available studies do not explain the genotype x dietary zinc interaction in our study. The reported studies used lean animals or humans to test the effect of zinc on leptin concentrations in either *in vivo* or *in vitro* systems. Our result was observed in genetically obese rats. Although DIO mice are genetically prone to develop

obesity, environmental factors, such as diet, are needed to express the genotype. Another reason for the different results is that the zinc-supplemented diet had a higher amount of zinc (150 ppm) compared to the zinc supplementation with 20 mg/l or 20 ppm in the *in vivo* studies. It is possible the zinc amount used in our study may have attenuated leptin production such as that observed *in vitro* by Chen et al. (2001) where the high (1 mM) zinc dose attenuated, while the low (0.1 mM) zinc dose augmented leptin production of STZ mouse adipocytes. More investigations would provide greater insight in this area.

Serum Free Fatty Acids and Triglycerides

Our observation of fa/fa having higher serum FFA and TG concentrations compared to lean Zucker rats (*Tables 21 and 22*) is supported by Guesnet and colleagues (1990). We did not observe a significant zinc effect on serum FFA or TG concentrations in either lean or fa/fa Zucker rats. To our knowledge, the effect of dietary zinc on serum FFA and triglyceride concentrations in lean and fa/fa Zucker rats has not been reported in the literature. Not observing an effect of dietary zinc on serum FFA and TG concentrations implies that zinc may not be involved in the enzymes involved in FFA and TG metabolism

Summary

Overall, dietary zinc concentrations (5, 30, 150 ppm) influenced serum leptin in fa/fa rats and pancreas zinc concentrations in both lean and fa/fa Zucker rats, but did not influence lipid metabolism (FFA and TG) and pancreatic function in both lean and fa/fa Zucker rats.

Immunohistochemistry

Insulin

To our knowledge, there is limited research that used insulin immunohistochemistry in combination with other parameters to determine pancreas function. Brown and colleagues (1999) used immunohistochemistry in one part of their experiments to determine chronic effects of a novel PPAR-γ agonist on pancreatic islet morphology and, in combination with other parameters, to determine pancreatic function. Buckingham and associates (1998) used insulin immunohistochemistry to observe insulin staining intensity and patterns, as well as morphology in fa/fa and lean Zucker rats receiving or not receiving chronic rosiglitazone therapy (another PPAR-γ agonist). In combination with plasma parameters (e.g. insulin, glucose) insulin immunohistochemistry was used to comment on the indirect effect of drug therapy on pancreatic function (Buckingham et al. 1998).

To our knowledge, both the Lipid and the Zinc studies are the first to examine insulin immunolocalization and islet patterns, thus providing an indication of pancreatic function, in response to dietary treatments in each respective study. Therefore, comparisons with other studies are limited and the interpretation is based mostly on speculations. However, the insulin immunostaining patterns of the fa/fa Zucker rats observed in our study are similar to that observed by Buckingham et al. (1998). The fa/fa pancreatic islets were enlarged, quite disorganized and expanded into the exocrine pancreas tissue. As also confirmed in the Buckingham et al (1998) study, the insulin staining pattern of fa/fa Zucker rats in both Lipid and Zinc studies was patchy, not uniform and not centralized in the islets.

Comparisons to our immunohistochemical staining results for lean Zucker rats are not found in the literature. The immunostaining pattern for lean Zucker rats revealed more organized and uniform insulin staining pattern that were more centralized within the islets. The pancreatic insulin immunostaining pattern of the lean Zucker rat possibly reflects the result of normal peripheral insulin sensitivity in this genotype. In contrast to the fa/fa rat, lean pancreatic β-cells do not need to undergo hypertrophy and hyperplasia to meet the insulin demands of the lean rat.

Lipid Study

As stated in the *Results* section, differences only in genotype were observed in the insulin immunostaining pattern of fa/fa and lean Zucker rats (*Figure 3*). There were no differences in dietary treatment groups for insulin immunostaining pattern or intensity. As stated in both the *Results* and *Discussion* sections, only differences due to genotype were observed in serum insulin, C-peptide concentrations, and serum glucose at t=15 and t=30 minutes of the OGTT. Along with the insulin immunohistochemistry, these results indicate pancreas β-cell function was impaired in the fa/fa rats compared to the lean Zucker rats of this study. The dietary treatments did not influence pancreatic function in either fa/fa and lean Zucker rats; therefore, at a level of 10% (w/w) fat in the diet, fatty acid composition (n-3 vs. n-6 or long vs. very long n-3 fatty acids) had no effect on pancreatic function.

It would be interesting if similar immunohistochemical results were obtained from rats fed diets with similar fatty acid compositions but at higher fat levels [e.g. 15, 20% (w/w)]. Mohan and colleagues (1991) found higher fasting serum insulin and glucose

concentrations in fa/fa Zucker rats fed 20% (w/w) safflower oil compared to fa/fa rats fed 20% (w/w) coconut or menhaden oil; the diets were not controlled for SAT, MUFA, and PUFA amounts. However, the researchers did not use immunohistochemistry to look at insulin staining patterns of rat pancreatic tissue, which may have given more insight on the pancreatic function of these animals. Higher fat amounts, as seen in the Mohan et al. (1991) study, more closely resemble both the fat recommendations and actual intake of North American humans (30% and 42% total calories from fat, respectively).

Zinc Study

Similar to the Lipid Study, genotype differences were observed in the insulin immunostaining pattern of fa/fa and lean Zucker rats (Figure 4). However, there were no differences among dietary treatment groups for insulin immunostaining pattern or intensity, indicating that dietary zinc has no effect on pancreatic insulin immunostaining. Combining this result with serum C-peptide concentration (*Tables 18 and 19*) and fasting serum insulin concentration (Petroulakis 2000), the dietary zinc treatments did not influence pancreatic function in both fa/fa and lean Zucker rats. It is possible the level of zinc supplementation may not have been high enough to influence pancreatic function. Although different animal studies were used, lower serum insulin concentrations were observed in studies by Simon (1998) (db/db mouse) and Bégin-Heick and colleagues (1985) (Sprague-Dawley rat) that used higher levels of dietary zinc supplementation (300 ppm and 964 ppm zinc, respectively). Unfortunately, these studies did not look at pancreatic insulin immunostaining patterns. However, it should be mentioned that higher

levels of dietary zinc may have negative consequences for copper status (L'Abbé et al. 1984).

Using another animal model, Tobia and associates (1998) found male Wistar BB rats fed diets of 1000 ppm zinc had pancreas tissue with nearly normal immunostaining for insulin and for other pancreatic endocrine hormones. Usually, male BB Wistar rats spontaneously develop type 1 DM around 100 days of age; however, a 60% reduction in the development of diabetes was observed in rats fed the high zinc diet. Therefore, it would be interesting to use insulin immunohistochemical techniques to help determine pancreatic function in animals fed diets with higher dietary zinc concentrations.

Metallothionein

Comparisons of our results to the literature are limited, with no published studies describing the effects of dietary fat on pancreatic MT staining intensity and immunolocalization in fa/fa Zucker rats or in any animal model. The number of zinc studies is few, with most based on observing pancreatic tissue after acute (one injection) doses of zinc. As well, all studies used lean animal models. The only dietary study that used immunohistochemistry for MT was by Ohly and colleagues (2000) who conducted a 22-week feeding trial by adding zinc sulfate to the drinking water of C57BL/6J and C57BL/6xSJL mice. Ohly and colleagues (2000) gave multiple doses of streptozotocin to induce diabetes. Besides this research, no studies have looked at the effect of dietary manipulation on metallothionein immunolocalization in the pancreas in genetic animal models of diabetes or obesity and insulin resistance. Therefore, to our knowledge, the Lipid and the Zinc Studies are the first to report the effects of dietary manipulation on

pancreatic metallothionein immunolocalization in the fa/fa Zucker rat, a genetic model of obesity and insulin resistance. Therefore, the discussion in this area will be based mostly on speculations.

It should also be mentioned, that to our knowledge, no studies reported and compared pancreatic MT immunostaining patterns in the lean and fa/fa Zucker rats. Pancreatic MT immunostaining patterns observed in both the Lipid and Zinc Studies were different than the immunostaining patterns of human and other animal models reported in the literature. In both the Lipid and Zinc studies, fa/fa rats had more intense pancreatic MT immunostaining compared to lean Zucker rats (*Tables 26 and 28*). As described in the *Results* section and the *Lipid* and *Zinc* subsections of this section, pancreatic MT immunolocalization was detected in the cytoplasm and nuclei of both acinar and peripheral islet cells of the fa/fa Zucker rat (*Tables 27 and 29*). With the exception of the lnZC rats, pancreatic MT immunolocalization in lean rats generally was detected in the cytoplasm and nuclei of only the acinar cells. The lnZC rats also had pancreatic MT immunolocalization in the cytoplasm and nuclei of the peripheral islet cells.

Pancreatic MT immunostaining results for the lean Zucker rats in both Lipid and Zinc Studies are in contrast to some published studies. As stated earlier, with the exception of lnZC rats in the Zinc Study, pancreatic immunostaining was detected in the acinar cells, but none was detected in the islet cells in lean Zucker rats of both our studies. Our results are in contrast to Andrews and colleagues (1990) who detected essentially no MT immunostaining in acinar cells but detected uniform MT immunostaining in islet cells of control adult male Sprague-Dawley rats. The authors,

however, did not indicate if uniform staining referred to all islet cells, central or peripheral islet cells. Wang and associates (1996) detected "infrequent and faint" MT immunostaining in both acinar and islet cells of control 7-week-old male Wistar rats. Again, the authors did not mention the pattern of MT immunostaining in the islet cells. Delisle and colleagues (1996) detected weak MT immunostaining in acinar cells and none in the islet cells of transgenic mice that overproduce MT-1. However, Ohly and associates (2000) detected uniform MT immunostaining in the cytoplasm of pancreatic islets cells of C57BL/6 and (C57BL/6xJJL)F_1 mice. In human studies using normal, pancreatic ductular carcinoma, or endocrine neoplastic pancreas samples, weak MT immunostaining was detected in the islet cells (Ohshio et al. 1996, Tomita 2000, Tomita and Matsubara 2000) with more intense MT staining observed in the pancreatic β-cells (Tomita 2000 and Tomita and Matsubara 2000).

Differences in reported results may be due to several factors that include the use of animal models or humans, age of the animals and experimental designs, and differences of pancreatic MT immunolocalization of animals compared to humans. As well, different MT immunostaining techniques may also contribute to differences in MT immunostaining detection. As discussed in the *Methods and Materials* Section, pancreata from both studies were fixed in 10% phosphate buffered formalin, embedded in paraffin and then tissue sections were cut. Andrews and associates (1990) fixed pancreas samples in PBS/formalin-like solutions, used embedding solutions and froze the samples prior to cutting tissue sections, while other studies cut sections from tissue that was fixed in embedding solutions only (Delisle et al. 1996, Tomita 2000, Tomita and Matsubara 2000). Ohly and colleagues (2000) first froze, sectioned and then acetone-fixed the

pancreatic tissue. Different pancreas preparation techniques may influence the structure of the protein of interest (MT), encourage more or less cross-linking with MT, thus influencing the immunodetection of MT by the primary antibody.

In addition, most published studies used polyclonal antibodies to MT-I and or to MT-II that were either raised in the laboratory where the study was conducted (Delisle et al. 1996), or donated from another laboratory (Andrews et al. 1990, Tomita 2000, Tomita and Matsubara 2000). Ohshio and associates (1996) used a monoclonal antibody to help localize MT in human normal or cancerous pancreas tissue. Our MT immunostaining technique used a manufactured monoclonal antibody (Dako) that detected both MT-I and MT-II.

Polyclonal antibodies come from several B-lymphocytes, whereas monoclonal antibodies come from one particular B-lymphocyte (Harlow and Lane 1988). Each B-lymphocyte produces an antibody against an epitope or a component of an antigen molecule (e.g. protein) of interest (Harlow and Lane 1988). Therefore, polyclonal antibodies help in the detection of an antigen, since collectively the antibodies will recognize many epitopes of the antigen. This is helpful when the antigen is bound to other proteins, carbohydrates, etc. that may "cover" some of its epitopes, thus preventing antibody recognition of those epitopes (Harlow and Lane 1988). The disadvantage of using polyclonal antibodies is there is possible cross-reactivity with similar epitopes of other antigens (Harlow and Lane 1988). Using monoclonal antibodies greatly limits cross-reactivity, as they are very specific for one epitope (Harlow and Lane 1988). Using monoclonal antibodies generates confidence that the immunostaining observed is the antigen under study. However, there is a danger of the monoclonal antibody not

detecting the epitope if it is bound to other molecules, especially if the tissue has been fixed for too long (Harlow and Lane 1988). To prevent this, fixing small tissue sections, using minimal fixation times, and tissue preparation (heat, enzymes) help ensure highly effective monoclonal antibody detection of the antigen of interest (Harlow and Lane 1988).

Small pancreatic tissue samples of both the Zinc and Lipid studies were fixed using the minimal fixing times possible (48 hours) prior to being embedded into paraffin. The pancreatic tissue sections were not treated with heat or with enzymes prior to immunohistochemical procedures, which might have improved MT immunodetection. Heat treatment was tried in our preliminary work; however, the treatment was discontinued, as the tissue samples were fragile and it was difficult to determine a satisfactory fixation time that would not destroy the tissue structure. However, both intense and less intense MT immunostaining was observed using the procedure outlined in the *Materials and Methods*, indicating the epitope was easily recognized by the monoclonal antibody that was used. As noted earlier, no published studies were found on the pancreatic MT immunostaining pattern of fa/fa or lean Zucker rats. Thus, the staining pattern observed in both the Lipid and Zinc Studies could be characteristic of this animal model.

Lipid Study

Compared to the lean rats, fa/fa Zucker rats had more intense MT staining (*Table 26*). The MT immunolocalization (mostly in the acinar cells) suggests high need for zinc by the acinar cells of fa/fa Zucker rats. Acinar cells synthesize and secrete some

pancreatic enzymes (e.g. carboxypeptidase A) that require zinc for their activity. To reiterate, hyperphagia is a characteristic of this genotype (Bray 1977) and the fa/fa rats of this study were no exception. The fa/fa rats significantly had higher daily feed intake compared to lean rats (*Tables 7 and 8*). Although pancreatic juice, digestive enzymes and pancreatic MT were not measured, the more intense MT immunostaining of the fa/fa rats suggest more enzymatic production which may have induced more MT production in the acinar cells. As well, studies observing rat pancreata 24 hours after one zinc injection showed more MT immunodetection in pancreatic acini of these animals. Because the fa/fa rats ate more feed, proportionally, they also received more dietary zinc than the leans, which may have induced higher MT synthesis causing greater immunodetection of this protein. The pair-weighed fa/fa rats that had some restriction in feed amounts to achieve similar body weights as the lowest treatment group did not vary in MT staining intensity (data not shown). It would be interesting to study the MT staining intensity in fa/fa rats that are fed the same feed amounts as lean Zucker rats.

The detection of MT in the fa/fa, but not lean Zucker rat peripheral islets is interesting (*Table 27*). It is possible the immunostaining may be in acinar cells that are enclosed and "infiltrated" by islets of the fa/fa rat. Most fa/fa islets are large, with poorly defined shapes. At times it was difficult to distinguish between acinar and islet cells because the islet boundary was not well defined. Therefore, future techniques having immunostaining for both MT and β-cells (and other endocrine cell immunostaining) concomitantly on the same tissue section need to be considered to help better distinguish between acinar and different islet cells.

Dietary treatment did not influence both MT immunostaining or immunolocalization pattern for both lean and fa/fa Zucker rats of this study. These

results imply that dietary fatty acid composition in a diet containing 10% (w/w) fat does not influence MT staining intensity or immunolocalization. No studies were found in the literature to either confirm or challenge our findings. Past studies indicate that many metals (e.g. zinc, copper), stress hormones, cytokines and metabolites made during stress and inflammation can induce MT (reviewed in Coyle et al. 2002). Although our immunohistochemical results indicated that dietary lipid does not induce pancreatic MT, it would have been interesting to determine if dietary lipid influenced MT concentration and MT-mRNA concentrations in the pancreas. As well, this study did not use a high level of dietary fat. It would be interesting to test the effects of dietary lipid on MT immunostaining and immunolocalization in Zucker rats fed diets with higher fat amounts.

Zinc Study

Similar to the observations of the Lipid Study, fa/fa rats had more intense MT immunostaining than the lean rats (*Table 28*). There was also a dietary effect where both faZD and lnZD rats had less intense MT immunostaining compared to ZC and ZS rats. Less intense MT immunostaining in the ZD rats was expected as zinc deficiency has been shown to dramatically decrease MT concentrations in the rat pancreas (Onosaka et al. 1982). As well, the pancreas is very sensitive to zinc intake and is believed to be involved in zinc homeostasis (De Lisle et al. 1996). The ZD rats also had lower pancreas zinc concentrations compared to the ZC and ZS rats (*Table 25*). Therefore, less MT immunostaining intensity closely paralleled the reduced pancreas zinc concentrations of the ZD rats.

It was unexpected that no differences were observed in MT immunostaining intensity between the ZC and the ZS rats. However, these results did coincide with the pancreas zinc concentrations, as there were no differences observed in the pancreatic zinc concentrations of the ZC and ZS rats. It was expected the ZS rats would have the most intense MT immunostaining, as high concentrations of zinc will induce the synthesis of MT. Ohly and colleagues (2000) similarly found no difference in MT immunostaining intensity in C57BL/6 and (C57BL/6 x SLJ)F_1 hybrid mice given Zn^{2+} drinking water (25 mmol/L Zn^{2+}) compared to mice given water not supplemented with zinc in addition to regular mouse chow. The zinc supplemented diet in our study had 150 ppm zinc, which is 5 times the amount suggested by Reeves and colleagues (1993), but it was not associated with copper deficiency as determined by unchanged liver copper concentrations (Petroulakis 2000). As no differences were detected between the ZC and ZS for the pancreas and pancreatic MT immunostaining intensity, it is possible the level of zinc supplementation was not high enough to cause significant differences to occur between the two dietary groups.

Similar MT immunolocalization patterns were observed between lean and fa/fa Zucker rats as observed in the Lipid study (*Table 29*). MT immunostaining was detected in the nuclei and cytoplasm of both acinar and peripheral islet cells in the fa/fa rats and in the nuclei and cytoplasm of only the acinar cells of the lean rats. However, MT immunostaining also was detected in peripheral islet cells of lnZC rats. It is not known why a similar pattern was not observed in lnZS rats.

VI. Summary and Conclusions

Major Research Findings

Lipid Study

- Dietary oil mixture treatment did not influence obesity in fa/fa Zucker rats, as seen in body and fat pad weights.
- The menhaden oil mixture treatment, which has higher amounts of very long chain n-3 fatty acids primarily from EPA and DHA, lowered fasting serum free fatty acid, but not fasting serum triglyceride concentrations, in both fa/fa and lean Zucker rats.
- The safflower oil diet, which has long n-6 fatty acids primarily from linoleic acid, lowered the fasting serum insulin concentrations in both fa/fa and lean Zucker rats.
- Dietary oil mixture treatment did not attenuate hyperinsulinemia or hyperleptinemia in fa/fa Zucker rats.
- Dietary oil mixture treatment did not influence overall oral glucose tolerance in fa/fa Zucker rats.
- The fa/fa rats had more insulin immunostaining and more islets than the lean Zucker rats.
- The fa/fa rats had more intense metallothionein immunostaining located in the nuclei and cytoplasm of both acinar and islet cells. In lean rats, metallothionein was immunolocalized only in the nuclei and cytoplasm of the acini, with more intense staining occurring in lean rats fed the menhaden oil mixture and safflower oil mixture diets.

- Dietary oil mixture did not influence pancreatic function in either fa/fa and lean Zucker rats as observed in oral glucose tolerance tests, fasting serum C-peptide concentrations, C-peptide/insulin ratio and indirectly through insulin immunohistochemistry.
- Overall, in moderate (10% w/w) fat diets with controlled SAT, PUFA, MUFA amounts as well as similar PUFA:SAT ratio, long (ALA) and very long (EPA and DHA) n-3 fatty acids did not influence obesity, hyperinsulinemia and pancreatic function in the fa/fa Zucker rat.

Zinc Study

- The fa/fa rats fed the zinc supplemented (150 ppm) diet had 31% and 24% lower fasting serum leptin concentrations compared to fa/fa rats fed the zinc deficient (5 ppm) and the zinc control (30 ppm) diets, respectively, despite no changes in body weight or body fat.
- Fasting serum free fatty acid and triglyceride concentrations were unchanged in fa/fa Zucker rats fed zinc deficient (5 ppm) or zinc supplemented (150 ppm) diet.
- Rats fed the zinc deficient (5 ppm) diet had lower pancreas zinc concentrations, as well as lower total and relative pancreas zinc compared to rats fed the zinc control (30 ppm) and zinc supplemented (150 ppm) diets.
- The fa/fa rats had more insulin immunostaining and more islets than the lean Zucker rats.
- The fa/fa rats had more intense metallothionein immunostaining located in the nuclei and cytoplasm of both acinar and islet cells. Less intense metallothionein

immunostaining was observed in lean and fa/fa Zucker rats fed the zinc deficient (5 ppm) diet.

- Dietary zinc (5, 30, 150 ppm) did not influence pancreatic function as observed in fasting serum C-peptide concentrations, C-peptide/insulin ratio and indirectly through insulin immunohistochemistry.
- Overall, dietary zinc concentrations (5, 30, 150 ppm) influenced serum leptin in fa/fa rats and pancreas zinc concentrations in both lean and fa/fa Zucker rats, but did not influence lipid metabolism (FFA and TG) and pancreatic function in both lean and fa/fa Zucker rats.

Implications for Future Research

- Enzyme (activity) assays of lipoprotein lipase in muscle, blood vessels and adipose tissue, and hormone-sensitive lipase.
- Pancreas triglyceride concentrations and lipid profiles.
- To further characterize dynamic pancreatic β-cell function, measure both serum insulin and C-peptide during the oral glucose tolerance test.
- Frequently sampled intravenous glucose tolerance test to directly measure the secretory capacity of pancreatic β-cells.
- Performing meal-fed glucose tolerance test using a liquid meal replacement such as Ensure. This test would give a broader picture on the effects of glucose, as well as protein (amino acids) and fat (fatty acids) on pancreas response during a meal.
- Northern analyses OB-mRNA in various adipose depots (epididymal, perirenal and retroperitoneal).

- Determination of MT protein concentrations and Northern analyses of MT mRNA levels in pancreatic tissue.
- Adipose, muscle and hepatic lipid profiles and serum cholesterol.
- Nutrient interactions between dietary zinc and dietary oil mixtures.

VII. References

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APPENDIX A.

Lipid Study - Fatty Acid Profiles of Actual Menhaden Oil Mixture Diet at Different Stages and Diet Left in Food Cups for 24 Hours[1,2].

	MO Wk2	MO Wk4	MO Wk15	MO Wk16/17	MO Cup-20/6/01	MO Cup-27/6/01
C8:0						
X						
X						
C10:0						
C12:0	0.13	0.08	0.08	0.08	0.04	0.08
X	0.05	0.04	0.05		0.03	0.04
X		0.05	0.05	0.06	0.03	0.05
C14:0	5.09	5.14	5.11	5.14	5.09	5.02
X						
C14:1	0.05	0.05	0.05	0.05	0.05	0.05
pufa	0.25	0.24	0.26	0.25	0.24	0.24
X	0.08	0.04			0.03	0.03
X		0.08	0.08	0.08	0.08	0.08
C15:0	0.48	0.48	0.49	0.49	0.49	0.47
X	0.1	0.1	0.1	0.11	0.11	0.1
C16:0	14.44	14.51	14.59	14.47	14.46	14.3
C16:1 n9	0.32	0.32	0.32	0.32	0.32	0.32
pufa	0.15	0.16	0.16	0.16	0.15	0.15
C16:1 n7	6.83	6.9	6.9	6.94	6.86	6.78
pufa	0.19	0.2	0.21	0.21	0.2	0.19
X	0.24	0.24	0.25	0.25	0.25	0.24
X	0.23	0.23	0.23	0.24	0.24	0.23
X	1.05	1.07	1.06	1.07	1.06	1.05
X	0.17	0.19	0.18	0.18	0.17	0.17
C17:0	1.02	1.03	1.03	1.04	1.03	1.02
X	0.54	0.52	0.5	0.51	0.49	0.54
X	0.29	0.28	0.3	0.3	0.3	0.14
X	0.14	0.14	0.14	0.14	0.14	0.16
C17:1	0.52	0.52	0.52	0.53	0.53	0.53
pufa	0.14	0.14	0.14	0.15	0.15	0.14
X	0.02	0.04	0.04	0.04	0.04	0.04
X		0.04	0.04	0.04	0.04	

Lipid Study - Fatty Acid Profiles of Actual Menhaden Oil Mixture Diet at Different Stages and Diet Left in Food Cups for 24 Hours[1,2] (Cont'd)

	MO Wk2	MO Wk4	MO WK15	MO Wk16/17	MO Cup-20/6/01	MO Cup-27/6/01
C18:0	3.14	3.17	3.25	3.26	3.22	3.12
C18:1 n9	9.34	9.36	9.39	9.45	9.33	9.3
C18:1 n7	2.28	2.29	2.29	2.29	2.31	2.3
pufa	0.09	0.08	0.09	0.09	0.09	0.09
X	0.09	0.09	0.1	0.1		0.09
C18:2 n6	23.99	24.15	24.17	23.67	23.99	23.85
X						
X						
C18:3 n6	0.21	0.21	0.22	0.22	0.21	0.39
pufa	0.18				0.18	
X	0.36	0.36	0.35	0.36	0.36	0.35
C18:3 n3	1.17	1.16	1.14	1.14	1.13	1.11
X						
X						
X						
C18:4 n3?	2.2	2.22	2.25	2.25	2.23	2.22
poly	0.19	0.2	0.2	0.2	0.2	0.19
X	0.07	0.07	0.07	0.04	0.03	0.07
X	0.05	0.05	0.05	0.07	0.04	
C20:0	0.26	0.26	0.27	0.27	0.28	0.26
X	0.09	0.09	0.09	0.09	0.11	0.09
C20:1 n9	0.85	0.85	0.86	0.87	0.87	0.84
X						
C20:1 n7?	0.2	0.2	0.2	0.21	0.23	0.21
poly	0.06	0.05			0.08	0.06
X	0.16	0.16	0.17	0.17	0.18	0.16
C20:2 n6	0.05	0.04	0.05	0.06	0.06	0.04

Lipid Study - Fatty Acid Profiles of Actual Menhaden Oil Mixture Diet at Different Stages and Diet Left in Food Cups for 24 Hours[1,2] (Cont'd)

	MO Wk2	MO Wk4	MO Wk15	MO Wk16/17	MO Cup-20/6/01	MO Cup-27/6/01
C20:3 n6	0.17	0.17	0.17	0.17	0.18	0.17
X	0.17	0.16	0.16	0.16	0.16	0.16
C20:4 n6	0.53	0.52	0.52	0.51	0.5	0.51
pufa	0.12	0.12	0.12	0.16	0.12	0.16
C20:3 n3	0.19	0.19	0.2	0.2	0.19	0.32
C20:4 n3?	1.23	1.22	1.22	1.21	1.21	1.22
C20:5 n3	7.76	7.73	7.69	7.75	7.61	7.8
X	0.17	0.17	0.18	0.18	0.18	0.18
C22:0	0.13	0.13	0.13	0.12	0.14	0.12
X						
X						
C22:1 n9	0.09	0.1	0.09	0.1	0.1	0.09
pufa		0.03			0.03	
C21:5 n3?	0.41	0.4	0.39	0.42	0.39	0.41
pufa	0.14	0.14	0.13	0.14	0.14	0.14
X	0.04	0.11	0.1	0.1	0.1	0.09
X	0.34	0.33	0.32	0.35	0.34	0.35
X	0.1	0.1	0.1	0.12	0.11	0.11
X		0.04	0.04	0.05	0.05	
C22:5 n3	1.53	1.47	1.48	1.49	1.5	1.55
C22:6 n3	8.75	8.44	8.4	8.49	8.49	8.89
C24:0	0.08	0.08	0.08	0.09	0.1	0.09
C24:1 n9	0.26	0.25	0.25	0.28	0.27	0.25
pufa						
Others	0.21	0.22	0.12	0.23	0.34	0.5
Total	99.97	100.01	99.98	99.98	100	100.01

Lipid Study - Fatty Acid Profiles of Actual Menhaden Oil Mixture Diet at Different Stages and Diet Left in Food Cups for 24 Hours[1,2] (Cont'd)

	MO Wk2	MO Wk4	MO Wk15	MO Wk16/17	MO Cup-20/6/01	MO Cup-27/6/01
SAT	25.98	26.1	26.26	26.17	26.06	25.6
MUFA	20.74	20.84	20.87	21.04	20.87	20.67
PUFA	53.04	52.85	52.73	52.54	52.73	53.24
Total	99.76	99.79	99.86	99.75	99.66	99.51
n-6	29.63	29.85	29.78	29.41	29.8	29.54
n-3	23.24	22.83	22.77	22.95	22.75	23.52
VLC n-3	19.87	19.45	19.38	19.56	19.39	20.19
EPA+DHA	16.51	16.17	16.09	16.24	16.1	16.69
PUFA/SAT	2.04	2.02	2.01	2.01	2.02	2.08
n-6/SAT	1.14	1.14	1.13	1.12	1.14	1.15
LA/SAT	0.92	0.93	0.92	0.90	0.92	0.93
n-6/n-3	1.27	1.31	1.31	1.28	1.31	1.26
LA/ALA	20.50	20.82	21.20	20.76	21.23	21.49

[1]Fatty acid values are reported in percent of dietary oil mixture. Percent values are reported as means.

[2] MO = Menhaden oil mixture.

APPENDIX B.

Lipid Study – Fatty Acid Profiles of Actual Safflower Oil Mixture Diet at Different Stages and Diet Left in Food Cups for 24 Hours[1,2].

	SO Wk2	SO Wk4	SO Wk15	SO Wk16/17	SO Cup-20/6/01	SO Cup-27/6/01
C8:0	1.55	1.56	1.6	1.56	1.57	1.57
X	0.13	0.15				
X	0.07	0.08				
C10:0	1.18	1.19	1.22	1.2	1.21	1.21
C12:0	8.74	8.82	8.87	8.82	8.89	8.86
X	0				0.06	
X						
C14:0	3.45	3.47	3.46	3.45	3.47	3.48
C14:1 pufa						
X						
X						
C15:0	0					
X						
C16:0	7.37	7.36	7.38	7.32	7.36	7.42
C16:1 n9 pufa	0.1	0.09	0.09	0.09	0.1	0.1
C16:1 n7 pufa						
X						
X						
X						
X						
C17:0	0.16	0.14	0.11	0.1	0.12	0.13
X						
X						
X						
C17:1 pufa						
X						
X						

Lipid Study – Fatty Acid Profiles of Actual Safflower Oil Mixture Diet at Different Stages and Diet Left in Food Cups for 24 Hours[1,2].

	SO Wk2	SO Wk4	SO Wk15	SO Wk16/17	SO Cup-20/6/01	SO Cup-27/6/01
C18:0	2.56	2.56	2.63	2.55	2.57	2.58
C18:1 n9	16.93	16.9	16.92	16.91	16.9	17.03
C18:1 n7	0.84	0.83	0.84	0.83	0.83	0.85
pufa X						
C18:2 n6	54.83	54.68	54.64	54.6	54.49	54.58
X	0.09	0.1	0.09	0.1	0.11	0.09
X						
C18:3 n6						
pufa X						
C18:3 n3	0.88	0.87	0.87	0.87	0.87	0.87
X	0.04	0.05	0.06	0.06	0.07	0.04
X	0.1	0.06	0.07	0.07	0.08	0.05
X		0.11	0.12	0.13	0.14	0.11
C18:4 n3?						
poly X						
X						
C20:0	0.32	0.32	0.32	0.35	0.36	0.32
X						
C20:1 n9	0.28	0.28	0.28	0.34	0.35	0.28
X					0.05	0.04
C20:1 n7?						
poly X						
C20:2 n6						

Lipid Study – Fatty Acid Profiles of Actual Safflower Oil Mixture Diet at Different Stages and Diet Left in Food Cups for 24 Hours[1,2].

	SO Wk2	SO Wk4	SO Wk15	SO Wk16/17	SO Cup-20/6/01	SO Cup-27/6/01
C20:3 n6						
X						
C20:4 n6						
pufa						
C20:3 n3						
C20:4 n3?						
C20:5 n3						
X						
C22:0	0.2	0.2	0.2	0.22	0.22	0.21
X						
X						
C22:1 n9						
pufa						
C21:5 n3?						
pufa						
X						
X						
X						
X						
C22:5 n3						
C22:6 n3						
C24:0	0.09	0.09	0.1	0.1	0.1	0.1
C24:1	0.11	0.1	0.1	0.1	0.1	0.08
n9						
pufa						
Others				0.23		0.16
Total	100.02	100.01	99.97	100	100.02	100.16

Lipid Study – Fatty Acid Profiles of Actual Safflower Oil Mixture Diet at Different Stages and Diet Left in Food Cups for 24 Hours[1,2].

	SO Wk2	SO Wk4	SO Wk15	SO Wk16/17	SO Cup-20/6/01	SO Cup-27/6/01
SAT	25.82	25.94	25.89	25.67	25.93	25.88
MUFA	18.26	18.2	18.23	18.27	18.33	18.38
PUFA	55.94	55.87	55.85	55.83	55.76	55.74
Total	100.02	100.01	99.97	99.77	100.02	100
n-6	54.92	54.78	54.73	54.7	54.6	54.67
n-3	1.02	1.09	1.12	1.13	1.16	1.07
VLC n-3	0	0	0	0	0	0
EPA+DHA	0	0.00	0.00	0.00	0.00	0.00
PUFA/SAT	2.17	2.15	2.16	2.17	2.15	2.15
n-6/SAT	2.13	2.11	2.11	2.13	2.11	2.11
LA/SAT	2.12	2.11	2.11	2.13	2.10	2.11
n-6/n-3	53.84	50.26	48.87	48.41	47.07	51.09
LA/ALA	62.31	62.85	62.80	62.76	62.63	62.74

[1]Fatty acid values are reported in percent of dietary oil mixture. Percent values are reported as means.

[2] SO = Safflower oil mixture.

APPENDIX C.

Lipid Study – Fatty Acid Profiles of Actual Flaxseed Oil Mixture Diet at Different Stages and Diet Left in Food Cups for 24 Hours[1,2].

	FXO Wk2	FXO Wk4	FXO Wk15	FXO Wk16/17	FXO Cup-20/6/01	FXO Cup-27/6/01
C8:0	1.54	1.57	1.57	1.56	1.56	1.51
X	0.12	0.14				
X	0.07	0.08				
C10:0	1.18	1.2	1.22	1.2	1.21	1.17
C12:0	8.77	8.91	8.9	8.9	8.88	8.75
X				0.04	0.07	
X						
C14:0	3.42	3.45	3.45	3.44	3.44	3.44
X						
C14:1 pufa						
X						
X						
C15:0						
X						
C16:0	6.35	6.37	6.39	6.35	6.35	6.41
C16:1 n9 pufa	0.08	0.08		0.08	0.08	
C16:1 n7 pufa			0.08		0.13	0.08
X						
X						
X						
X						
C17:0	0.16	0.16	0.12	0.11	0.13	0.14
X						
X						
X						
C17:1 pufa						
X						
X						

Lipid Study – Fatty Acid Profiles of Actual Flax Seed Oil Mixture Diet at Different Stages and Diet Left in Food Cups for 24 Hours[1,2].

	FXO Wk2	FXO Wk4	FXO Wk15	FXO 16/17	FXO Cup-20/6/01	FXO Cup-27/6/01
C18:0	3.03	3.06	3.11	3.05	3.07	3.06
C18:1 n9	18.06	18.06	18.13	18.12	18.1	18.29
C18:1 n7	0.82	0.83	0.83	0.85	0.85	0.84
pufa X						
C18:2 n6	19.22	19.15	19.24	19.23	19.18	19.32
X	0.05	0.05	0.05	0.05		0.05
X	0.23	0.25	0.24	0.24	0.25	0.23
C18:3 n6						
pufa X						
C18:3 n3	35.74	35.4	35.58	35.55	35.45	35.63
X	0.09	0.04		0.04	0.05	
X		0.14	0.12	0.12	0.12	0.1
X		0.04		0.04	0.05	
C18:4 n3?						
poly X						
X						
C20:0	0.23	0.24	0.24	0.24	0.26	0.23
X						
C20:1 n9	0.27	0.28	0.27	0.28	0.29	0.25
X	0.02	0.03			0.05	0.05
C20:1 n7?						
poly X						
C20:2 n6						

Lipid Study – Fatty Acid Profiles of Actual Flax Seed Oil Mixture Diet at Different Stages and Diet Left in Food Cups for 24 Hours[1,2].

	FXO Wk2	FXO Wk4	FXO Wk15	FXO Wk16/17	FXO Cup-20/6/01	FXO Cup-27/6/01
C20:3 n6						
X						
C20:4 n6						
pufa						
C20:3 n3						
C20:4 n3?						
C20:5 n3						
X						
C22:0	0.12	0.12	0.12	0.13	0.13	0.12
X	0.11	0.09	0.08	0.08	0.1	0.08
X	0.21	0.19				
C22:1 n9						
pufa						
C21:5 n3?						
pufa						
X						
X						
X						
X						
C22:5 n3			0.19	0.17	0.19	0.18
C22:6 n3						
C24:0	0.07	0.08	0.07	0.07	0.07	0.07
C24:1 n9	0.02					
pufa						
Others				0.04	0.06	
Total	99.98	100.01	100	99.98	100.12	100

Lipid Study – Fatty Acid Profiles of Actual Flax Seed Oil Mixture Diet at Different Stages and Diet Left in Food Cups for 24 Hours[1,2].

	FXO Wk2	FXO Wk4	FXO Wk15	FXO Wk16/17	FXO Cup-20/6/01	FXO Cup-27/6/01
SAT	25.38	25.66	25.27	25.17	25.27	24.98
MUFA	19.27	19.28	19.31	19.33	19.5	19.51
PUFA	55.33	55.07	55.42	55.44	55.29	55.51
Total	99.98	100.01	100	99.94	100.06	100
n-6	19.5	19.45	19.53	19.52	19.43	19.6
n-3	35.83	35.62	35.89	35.92	35.86	35.91
VLC n-3	0	0	0.19	0.17	0.19	0.18
EPA+DHA	0	0	0	0	0	0
PUFA/SAT	2.18	2.15	2.19	2.20	2.19	2.22
n-6/SAT	0.77	0.76	0.77	0.78	0.77	0.78
LA/SAT	0.76	0.75	0.76	0.76	0.76	0.77
n-6/n-3	0.54	0.55	0.54	0.54	0.54	0.55
LA/ALA	0.54	0.54	0.54	0.54	0.54	0.54

[1]Fatty acid values are reported in percent of dietary oil mixture. Percent values are reported as means.

[2] FXO = Flax seed oil mixture.