THE EFFECTS OF DIETARY ZINC ON ADIPOCYTE GLUT 4 EXPRESSION, ADIPOSITY, HYPERINSULINEMIA AND ZINC STATUS OF THE OBESE (fa/fa) ZUCKER RAT

By
Kathy Petroulakis

A thesis submitted to the Department of Foods and Nutrition in partial fulfillment of the requirements for the degree of Master of Science

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THE UNIVERSITY OF MANITOBA

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ABSTRACT

THE EFFECTS OF DIETARY ZINC ON ADIPOCYTE GLUT 4 EXPRESSION, ADIPOSITY, HYPERINSULINEMIA AND ZINC STATUS OF THE OBESE (fa/fa) ZUCKER RAT

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Obesity is a major risk factor for insulin resistance and Type 2 diabetes. Subjects with obesity may demonstrate suboptimal zinc status and decreased levels of glucose transporter protein 4 (Glut 4). Both zinc and Glut 4 are involved with glucose homeostasis; however, the relationship between zinc and Glut 4 has not been studied.

The primary objective of this study was to investigate the effects of dietary zinc manipulation on the expression of total Glut 4 in adipocytes of lean and obese (fa/fa) Zucker rats. The study was further designed to investigate the effects of dietary zinc deficiency and supplementation on 1) body fat deposition (adiposity), 2) hyperinsulinemia and 3) zinc status of the Zucker rat. Weanling lean and fa/fa Zucker rats were fed zinc deficient (ZD, 5 ppm zinc), zinc control (ZC, 30 ppm zinc), or zinc supplemented (ZS, 150 ppm zinc) diets, or were pair-weighed (PW, 30 ppm zinc) for 9 weeks. At 15 weeks of age, rats were assessed for total Glut 4 expression in adipocytes, fat deposition (epididymal and perirenal fat pad weights; epididymal adipocyte diameter; liver lipid concentration), degree of insulin resistance (serum insulin and glucose concentrations) and tissue zinc concentrations (femur, serum and liver).

After the 9 week feeding trial, the fa/fa rats had heavier body and fat pad weights, larger adipocyte diameters, higher liver lipid concentrations, hyperinsulinemia, and higher femur and serum zinc concentrations compared to the lean rats. On the contrary, the fa/fa rats showed *lower* hepatic zinc concentrations and *lower* total Glut 4 expression

in adipocytes. Despite significant genotype differences in serum insulin, serum glucose concentrations were similar in lean and fa/fa rats. Dietary zinc level did not have a significant effect on body weight, fat pad weight, adipocyte diameter or liver lipid concentration. Zinc supplementation in both lean and fa/fa rats resulted in higher femur zinc concentrations (45.2% and 37.2%, respectively) compared to rats fed a zinc deficient diet. Zinc supplementation in lean rats elevated (16.5%) serum zinc concentration compared to lean rats fed a zinc deficient diet, but this was not observed in the fa/fa genotype. Hepatic zinc concentration was lower in the fa/fa rats compared to the lean rats, irrespective of dietary treatment.

Lean rats expressed more Glut 4 in adipocytes compared to fa/fa rats (1645 ± 15 vs. 995 ± 150 arbitrary units, respectively, $p<0.05$), and fa/fa rats fed a zinc supplemented diet had less adipocyte Glut 4 compared to all other (lean and fa/fa) treatment groups ($p<0.05$).

There are several conclusions in this study. Firstly, the results suggest that diet (zinc supplementation) combined with genetics (fa/fa) further reduces adipocyte Glut 4 expression in Zucker rats. There is also an indication that zinc supplementation does not aggravate body or hepatic fat deposition. Despite the role of zinc in insulin action, the results of this study suggest that dietary zinc supplementation (150 ppm) does not attenuate hyperinsulinemia in the fa/fa rat. Furthermore, it appears that the Zucker rat shows altered zinc metabolism and a zinc supplemented diet appears to have different effects on the short-term (serum) zinc status of lean rats compared to the fa/fa rats.

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I. Literature Review

Introduction

Diabetes mellitus is a chronic disease in which excessive quantities of glucose are retained in the bloodstream (hyperglycemia) (Kahn, 1994). In normal physiological conditions, the hormone insulin functions to regulate blood glucose concentrations. This homeostatic mechanism of insulin does not function properly in diabetes.

Two types of diabetes have been identified. Type 1 diabetes is characterized by high blood glucose concentrations secondary to an insufficiency of insulin. In type 2 diabetes, the adult-onset form of the disease and the focus of this thesis, hyperglycemia is a result of a resistance to the actions of insulin. Insulin resistance is the decreased ability or the reduced potency of insulin to (1) act on peripheral tissues (muscle and adipose) to stimulate glucose utilization and (2) inhibit hepatic glucose production (Kahn, 1994).

There is presently no cure for diabetes. Research in the prevention and treatment of type 2 diabetes has focused on elucidating the key risk factors of the disease and intervening in these early stages to ultimately prevent the disease.

Obesity is a key risk factor for insulin resistance and type 2 diabetes and the progression of obesity and insulin resistance is the focus of this thesis. Research in the prevention and treatment of obesity and its associated metabolic abnormalities has focused on the role of dietary intervention. Nutrition management of obesity and insulin resistance to ultimately prevent the development of overt diabetes has become an integral component of diabetes research. Studies have identified potential interrelationships between dietary zinc and glucose metabolism in this disease state. The purpose of this thesis is to investigate the effects of dietary zinc on 1) the expression of the glucose

transporter protein 4 (Glut 4), 2) adiposity, 3) hyperinsulinemia and 4) zinc status of the obese fa/fa Zucker rat. This literature review will address the current state of knowledge for these topic areas.

Type 2 Diabetes

a) Epidemiology of Type 2 Diabetes

Diabetes affects approximately 4% of the Canadian population but may reach up to 17% in some Native Canadian communities (Blanchard et al., 1996). By 1991, there were 47,890 Manitoba residents over 25 years of age who had diabetes and this yields a crude prevalence of 66.9/1000 (Blanchard et al., 1996). The prevalence of diabetes is increasing in many populations worldwide. With increasing availability of life-prolonging options, it can be anticipated that the prevalence of diabetes in Manitoba will continue to rise unless there is a dramatic decline in the incidence rate.

One of the difficulties in preventing type 2 diabetes is that it is a disease with significant genetic determinants and a clear genetic marker has not yet been identified. Type 2 diabetes is more prevalent among Native Americans than among American Caucasians, African-Americans or Hispanic-Americans (Bogardus and Lilloja, 1992). Twin studies have demonstrated a high concordance rate of the disease among monozygotic twins (60-90%) compared to dizygotic twins. Furthermore, a history of diabetes in a first-degree relative doubles the risk of diabetes while offspring of two diabetic parents have an 80% lifetime risk of diabetes (Bogardus and Lilloga, 1992).

The Pima Indians of Arizona have the highest reported prevalence of the disease of any population in the world (Bogardus and Lillioja, 1992). More than half of this

population over the age of 35 years has type 2 diabetes. The Pima Indians have become the focus of diabetes investigation and have provided information to help identify genetic determinants of type 2 diabetes in humans.

b) Pathogenesis of Type 2 Diabetes

The development of type 2 diabetes progresses through different stages (Knowler et al., 1995). The initial stage manifests itself as insulin resistance. Despite this impairment in insulin action, blood glucose concentrations remain normal. This normal glucose tolerance is secondary to an increased insulin secretion from the pancreatic β cells (Knowler et al., 1995). Thus, the trade-off for normal fasting glucose concentrations is compensatory hyperinsulinemia. When insulin secretion is no longer sufficient to compensate for insulin resistance, insulin deficiency arises and hyperglycemia worsens to the point of diabetes (Knowler et al., 1995).

The contribution of insulin resistance to the pathogenesis of type 2 diabetes is widely recognized. However, the precise etiology of insulin resistance is not understood. Obesity, particularly abdominal obesity, is causally related to the development of insulin resistance and is considered a key risk factor for the development of type 2 diabetes (Golay et al., 1994).

c) Environmental Influences on Type 2 Diabetes

Despite a clear genetic disposition to type 2 diabetes, environmental factors influence the expression of the disease. Both diet and lifestyle contribute to the development of obesity; a sedentary lifestyle and a high fat, high calorie diet has been linked to the development of type 2 diabetes (Bogardus and Lilloja, 1992).

Obesity affects 33% of the Canadian population and costs the health care system approximately 6 billion dollars each year (Golay et al., 1994). Body fatness is strongly correlated to insulin resistance; with excess weight gain, the body becomes markedly resistant to the action of insulin (Ravussin et al., 1996). Tissue sensitivity to insulin declines by 30-40% when an individual becomes 35-40% over their ideal body weight (Daly, 1994). Studies in Pima Indians have shown that adiposity is the most important predictor for the development of type 2 diabetes in children (Walker, 1995) and that weight loss is followed by significant increases in insulin sensitivity as demonstrated by increased glucose disposal (Ravussin and Swinburn, 1996). Although the exact causes of obesity are unknown, it is understood that obesity is partly a result of an imbalance between energy intake and energy expenditure. Of particular interest to this study is the cellular abnormalities in obesity and insulin resistance.

The Glucose Transporter Protein 4 (Glut 4)

There is a body of evidence in the literature that suggests decreased expression and function of the glucose transporter protein 4 (Glut 4) as a contributing factor to insulin resistance (Garvey, 1994).

The transport of glucose into cells is facilitated by a family of seven transport proteins (Glut 1 through 7) which exhibit marked differences in function and tissue-specific expression (Garvey, 1994). Glut 4 is predominantly found in the insulin responsive tissues, including muscle and adipose, and is the glucose transporter protein most commonly investigated in obesity and diabetes research. Under basal conditions (non-insulin stimulated) Glut 4 is predominantly localized to an intracellular vesicle

compartment (Pessin and Bell, 1992). In response to insulin, Glut 4 is translocated to the cell surface to facilitate the uptake of glucose from the blood to the tissue (muscle or adipose). In type 2 diabetes and insulin resistance, it has been proposed that the impairment of glucose uptake by tissues may be in part explained by a depletion of Glut 4 in this population group. The following sections will discuss investigations of Glut 4 expression in both human and animal models of obesity and type 2 diabetes. Fewer studies have addressed the effects of dietary intervention on the expression of Glut 4.

a) Glut 4 Expression in Humans

The results for Glut 4 expression in humans with obesity and/or diabetes are variable. It has been proposed that a *possible* explanation for altered glucose uptake in human subjects with obesity and insulin resistance/type 2 diabetes may be a decreased expression of Glut 4 in adipose and/or muscle tissue (Garvey et al., 1991). Based on the observations of Garvey et al. (1991), Glut 4 expression in adipocytes isolated from patients with obesity and type 2 diabetes is less than adipocyte Glut 4 expression observed in lean controls. Garvey et al. (1991) also identified that in obesity, there is a depletion in the adipocyte intracellular pool of Glut 4 compared to lean controls, in addition to an inverse relationship between Glut 4 expression and adipocyte size. Furthermore, the authors demonstrated that patients with type 2 diabetes have a more profound depletion of Glut 4 in both the intracellular and plasma membrane components compared to obese (without diabetes) subjects and healthy controls.

Glucose transport activity, Glut 4 mRNA and Glut 4 protein in adipocytes from lean, obese and patients with type 2 diabetes has also been investigated by Pessin and Bell (1992). The authors observed a decrease in adipocyte insulin stimulated glucose

transport activity in subjects with obesity and diabetes, which was associated with decreased levels of both Glut 4 mRNA and Glut 4 protein in adipocytes.

The findings of Garvey et al. (1991) for lower Glut 4 expression in adipocytes of patients with obesity and diabetes are supported by Dohm et al. (1991) for low Glut 4 expression in muscle. Dohm et al. (1991) demonstrated that Glut 4 expression in the rectus abdominus and the vastus lateralis muscle was significantly lower in patients with obesity (with or without associated diabetes) compared to healthy subjects. On the contrary, Pederson et al. (1990) demonstrated that there was similar expression of Glut 4 in vastus lateralis muscle of both obese patients and lean controls. Garvey (1994) also did not find differences in Glut 4 expression in these muscle tissues of patients with or without obesity.

Based on the literature, it appears that the conclusions for Glut 4 expression in adipocytes are more consistent compared to the results for Glut 4 expression in muscle. This thesis project focuses on total Glut 4 expression in adipocytes isolated from lean and obese (fa/fa) Zucker rats.

b) Glut 4 Expression in Animal Models

Koranyi et al. (1990) studied Glut 4 expression in muscle and adipose tissue of diabetic db/db mice and showed that the expression of Glut 4 in adipose tissue and quadricep muscle were not different from levels observed in lean littermates. On the contrary, studies in the obese (fa/fa) Zucker rat model, the animal model used in this thesis project, illustrated different results (Shmaya et al., 1997). Shmaya et al. (1997) showed that obese (fa/fa) Zucker rats have approximately one-third the Glut 4 content in epididymal adipocytes compared to lean controls. Interestingly, Glut 4 expression varies

according to the age of the Zucker rat. For example, Pederson et al. (1992) showed that 5 week and 10 week old fa/fa rats expressed more Glut 4 in adipocytes compared to lean controls. However, at 20 weeks of age, the expression of Glut 4 was significantly less in the fa/fa genotype compared to the lean controls.

According to Kahn et al. (1991), in streptozocin-induced diabetic rat models (animal model for type 1 diabetes), muscle Glut 4 levels are unaltered at 7 days of diabetes but are significantly reduced by 14 days, compared to non-diabetic controls.

c) Effects of Diet on Glut 4 Expression

Dietary intervention may affect the expression of Glut 4 in the muscle and/or adipose tissue of animals. High fat feeding has been shown to suppress the expression of Glut 4 in skeletal muscle of rats (Kahn and Pederson, 1993). Male Sprague Dawley rats fed a high fat diet (80% energy as fat) for 7 weeks showed a 34% reduction in Glut 4 levels in the quadricep muscle compared to rats fed a control diet (12% energy as fat). On the contrary to the findings of Kahn and Pederson (1993), Rosholt et al. (1994) demonstrated that rats fed a high fat diet (60% energy as fat) for 3 weeks showed no differences in muscle Glut 4 compared to rats fed a diet of 10% energy as fat. The differences in the results of Kahn and Pederson (1994) and Rosholt et al. (1994) may be attributed to the lower percentage of energy from fat and the shorter feeding study used by Rosholt et al. (1994).

Interestingly, high fat feeding may have tissue specific effects on Glut 4 expression. For example, Pederson et al. (1991) showed that Sprague-Dawley rats fed a high fat diet (80% energy as fat) for 7 weeks had a 81% reduction in adipocyte Glut 4 but only a 34% reduction in muscle Glut 4, in comparison to rats fed a control diet (12%

energy from fat). Nutrition intervention (high fat feeding) may therefore have more profound effects on adipocyte Glut 4 compared to muscle.

Ethanol is a dietary factor that may influence Glut 4 translocation to the cell membrane, but not total Glut 4 expression per se. Wilkes et al. (1996) demonstrated that Wistar rats fed a high ethanol diet (35% energy from ethanol) for 4 weeks had less translocation of Glut 4 to the membrane after insulin stimulation, compared to rats fed an ethanol-free diet.

Furthermore, diets supplemented with acarbose may influence Glut 4 expression (Dolan et al., 1997). Acarbose is a complex oligosaccharide that reversibly inhibits α-glucosidases present in the brush border of the small intestinal mucosa (Dolan et al., 1997). Hence, inhibition of these enzymes with acarbose feeding delays the absorption of glucose and thus results in a smaller rise in blood glucose concentrations after a carbohydrate meal. Dolan et al. (1997) demonstrated that lean and obese Zucker diabetic fatty rats (ZDF/Gmi-*fa* rats) fed a high acarbose diet (40 mg/100g chow) show elevated Glut 4 expression in gastrocnemius muscle compared to rats fed an acarbose-free diet.

Thus, there is evidence in the literature that nutritional factors may influence Glut 4 expression. However, the role of dietary zinc on the expression of Glut 4 has not been investigated in the literature, despite adequate literature supporting the role of zinc in obesity and diabetes/insulin resistance.

In addition to nutritional factors, exercise may also influence the expression of Glut 4. Rodnick et al. (1990) demonstrated that after 6 weeks of voluntary running in exercise-wheel cages, Sprague-Dawley rats had significantly more (60%) Glut 4 in plantaris muscle compared to control (non-exercised) rats. The effects of exercise on

Glut 4 expression in the Zucker rat have also been investigated (Etgen et al., 1997). Exercise trained obese Zucker rats exhibited significantly greater insulin-stimulated glucose transport activity in epitrochlearis muscles which was paralleled by a significant enhancement of insulin-stimulated cell surface Glut 4, compared to sedentary Zucker rats (Etgen et al., 1997). Thus it is possible that exercise may influence an increase in the translocation of Glut 4 to the cell membrane. Etgen et al. (1997) did not investigate the effects of exercise on Glut 4 expression in adipose tissue and to our knowledge, this area has not been investigated for the Zucker rat.

Zinc and Type 2 Diabetes

Zinc is a component of numerous biological enzymes including those of glucose metabolism (Chausmer, 1998). Zinc is also involved with the synthesis, storage and release of insulin (Tallman & Taylor, 1999). As part of the carboxypeptidase B enzyme, which converts proinsulin to insulin, zinc is involved with the activation of insulin. Furthermore, insulin is stored in the pancreatic β cell as a hexameric crystal containing a variable number of zinc molecules. The addition of zinc to insulin results in conformational changes (Goldman et al., 1974) and also enhances the effectiveness of insulin. In vitro studies have shown zinc to enhance insulin binding to adipocytes and to enhance lipogenesis (Coulsten et al. 1980). Given the role of zinc in carbohydrate metabolism and its functional and morphological relationship with insulin, it is suggested that zinc may play a role in the pathogenesis of type 2 diabetes. Experimental rats develop glucose intolerance after zinc deprivation (Hendricks and Mahoney, 1972). Other studies demonstrate that animal models and human subjects with diabetes show

zinc deficiency and this zinc deficiency may aggravate the insulin resistance in this disease state. However, whether zinc deficiency is a cause or consequence of diabetes remains unclear (Kahn, 1994).

a) Zinc Status and Type 2 Diabetes

Serum and tissue zinc concentrations are often used as markers of zinc status in both human and animal models of diabetes. The serum zinc concentration of genetically obese diabetic (db/db) mice is depressed compared to nondiabetic mice (Levine et al., 1983) whereas the femur zinc concentration of the db/db mouse is similar to lean controls (Simon, 1998). This is contrary to results for the obese (fa/fa) Zucker rat. Fa/fa rats have significantly higher femur zinc concentrations compared to lean controls (Donaldson et al., 1987). Studies in humans have demonstrated similar results. Niewoehner et al. (1986) found that 9% of the patients studied had serum zinc levels below normal (70 *u*g/dl). Sjorgen et al. (1988) also demonstrated that plasma zinc concentration is lower in subjects with diabetes, as compared with plasma zinc of healthy controls, and Kinlaw et al. (1983) demonstrated that 25% of patients have depressed serum zinc concentrations, although there was no significant difference between diabetic and control subjects. Other studies have reported that zinc levels in plasma of patients with type 2 diabetes were significantly lower than corresponding values in control subjects (Kumar and Rao, 1974; Pai and Prasad, 1988).

Dietary zinc restriction studies in humans have provided evidence that a depression in serum zinc is a late finding and may be reflecting late stage tissue deficiency (Prasad et al., 1978). Bone zinc concentration is an excellent representative

measure of total body zinc and is the tissue most often used to assess tissue zinc status (O'Leary et al., 1979).

The concentration of zinc in the femur of db/db and ob/ob mice is significantly lower than lean mice (Levine et al., 1983; Kennedy et al., 1986; Begin-Heick et al., 1985). Ob/ob mice also show significantly lower concentrations of zinc in liver, small intestine, pancreas, testes and smooth muscle compared to respective tissues from lean mice (Levine and Failla, 1987).

The exact mechanism for lower tissue zinc concentrations in animal models and human diabetes is unclear. Excess zinc excretion (hyperzincuria) may be a plausible reason, given that lower serum zinc and tissue zinc concentrations are accompanied by hyperzincuria. Urinary zinc excretion was markedly higher in diabetic mice compared to control mice and this was associated with lower serum and femur zinc concentrations (Levine et al., 1983). Db/db mice demonstrate significantly higher zinc excretion compared to lean controls (Simon, 1998). Hyperzincuria has also been noted in humans with diabetes (Kinlaw et al., 1980).

To reestablish optimal zinc status, dietary zinc supplementation may be an option. From the point of view of diabetes prevention and control, there is interest in the effects of zinc supplementation on slowing the progression of disease.

b) Zinc Supplementation and Type 2 Diabetes Mellitus

The clinical consequences of zinc deficiency in diabetic patients needs further documentation. Additional studies to provide a basis for zinc supplementation may serve beneficial in decreasing the morbidity of the disease. Zinc repletion could improve not only zinc status but may also improve insulin sensitivity in patients.

The effects of dietary zinc intake on zinc status have been investigated in experimental animal models of diabetes. Db/db mice fed a zinc adequate diet (10 ppm) demonstrate higher serum zinc concentration compared to mice fed a zinc deficient (2 ppm) diet for 12 weeks (Donaldson et al., 1988). Femur, renal and hepatic zinc concentrations were also higher in db/db mice fed a zinc adequate diet.

On the contrary to Donaldson et al. (1988), Southon et al. (1988) did not demonstrate a significant difference in femur and pancreatic zinc concentration when db/db mice were fed a zinc adequate diet (54 ppm) or a zinc deficient diet (1 ppm) for 27 days.

Other studies have investigated the effects of zinc supplementation. It is important to establish an appropriate level of supplementation. The recommended intake of zinc for mice and rats is 12 to 30 ppm or mg/kg diet (Reeves et al., 1993). Therefore, to observe an effect of zinc supplementation, exceeding the recommended intake. However, high ratios of dietary zinc to dietary copper have been reported to induce copper deficiency (L'Abbe & Fisher, 1984). Also, it is unclear how much zinc is consumed when drinking water is supplemented. Levine et al. (1983) supplemented the drinking water of db/db mice with 30 *u*g/ml zinc acetate for 4 weeks and did not find significantly altered serum or tissue zinc concentrations, although there was a tendency for higher zinc concentration in the serum and femur.

Simon (1998) demonstrated that db/db mice supplemented with 300 ppm zinc for 6 weeks had significantly higher femur zinc concentrations compared to zinc deficient (3 ppm) and zinc control (30 ppm) groups. There was also a trend towards higher serum zinc concentrations when db/db mice were supplemented with 300 ppm zinc. This level

of dietary zinc supplementation (300 ppm for 6 weeks) did not alter liver copper concentrations, an indicator of copper deficiency (Simon and Taylor, 2000).

In humans, short-term zinc supplementation (30 mg/day for 3 weeks) increased plasma zinc concentrations and the activity of 5'-nucleotidase, a zinc dependent enzyme, in 20 postmenopausal women with type 2 diabetes, compared to women given a placebo (Blostein-Fujii, 1997). However, the level of zinc supplementation used in this study (30 mg/day) is approximately twice the recommended nutrient intake for adult females. Furthermore, this study did not report the effects of zinc supplementation of diabetic or glycemic indices.

Zinc supplementation may also improve diabetic parameters in animals and humans. Zinc supplementation (1000 ppm) for 4 weeks reduced hyperglycemia and hyperinsulinemia in the ob/ob mouse (Begin-Heick et al., 1985). Simon (1998) demonstrated that dietary zinc supplemented (300 ppm) db/db mice had a significantly lower serum glucose concentration than the dietary zinc deficient group (3 ppm). Furthermore, db/db mice fed a low zinc diet (1 ppm) demonstrated significantly higher fasting blood glucose concentration than mice fed a zinc adequate diet (54 ppm) (Southon et al., 1988)

In humans, zinc supplementation of 220 mg oral zinc sulfate, three times daily for six to eight weeks, had no effect on glycosylated hemoglobin levels (an indicator of long term glycemic control) (Niewoehner et al., 1986). However, there was a significant increase in serum zinc concentrations of diabetic patients after supplementation compared to baseline serum zinc values.

Zinc and Adipose Tissue

The interactions of zinc with insulin action and potential benefits for glycemic control have received considerable attention (reviewed in Tallman & Taylor, 2000). However, the role of insulin as a lipogenic hormone in type 2 diabetes is often overlooked. In attempt to further our understanding of the benefits of zinc supplementation for glycemic control, the potential deleterious effect of zinc on obesity development, a major risk factor of type 2 diabetes, also need to be examined.

a) Zinc and Adipose Tissue Accretion

The suggestion that zinc supplementation may be detrimental to the development of obesity is based on the study of Chen et al. (1996). Dietary induced obese mice (DIO group, 80% energy from fat) had a higher body fat content (total carcass fat) after 3 weeks of the DIO diet, compared to mice fed a low fat diet (control group, 10% energy from fat). The addition of 200 ppm dietary zinc for 6 weeks further increased the body fat in the DIO group compared to the control group. The observations of Chen et al. (1996) suggest that the interaction between a high fat diet and zinc supplementation is deleterious from the point of view of obesity development. However, the percentage of energy from dietary fat (80% of total energy) is a confounding effect in this study; the increase in body fat may be secondary to the high fat and not necessarily from the zinc supplementation. Furthermore, the diet did not contain carbohydrate, and this may have contributed to altered lipid metabolism.

Nevertheless, Chen et al. (1996) also demonstrated that ob/ob mice fed a zinc supplemented (200 ppm) basal diet (10% energy from fat) for 6 weeks had significantly more body fat than ob/ob mice fed a marginally zinc deficient diet (4-6 ppm). Although

Chen et al. (1996) used a high fat diet in the previous experiment, zinc supplementation elevated body fat in genetically obese mice when the diet was 10% energy from fat.

The hypothesis that zinc supplementation may increase body fat is not supported by Simon (1998). Db/db mice fed a control diet (30 ppm zinc) for 6 weeks had significantly higher body weights than mice fed a zinc deficient diet (3 ppm) and zinc supplemented diet (300 ppm). Db/db mice on the control diet had a higher fat pad weight than the zinc deficient group, but there were no significant differences for fat pad weight between the zinc control and zinc supplemented group.

A possible explanation for increased body fat with zinc supplementation observed by Chen et al. (1996) may be the effect of zinc on increasing insulin sensitivity and therefore enhancing the role of insulin on lipogenesis.

b) Zinc, Insulin and Lipogenesis

Coulsten et al. (1980) investigated whether zinc alters the actions of insulin in vitro. The rate of lipogenesis in rat epididymal adipocytes was used as an index of the biological potency of insulin. This was accomplished by measuring the radioactivity of [^{3}H]-glucose incorporated into lipid, in epididymal adipocytes incubated with zinc alone, insulin alone or both insulin and zinc. In vitro incubation of adipocytes with 0.25, 0.5 and 1 mM zinc produced a significant increase in lipogenesis (144%, 1102% and 1548%, respectively). Adipocytes incubated with insulin alone at 5, 10 and 20 μU/ml also produced significant stimulation of lipogenesis (172%, 938% and 3547%, respectively). When adipocytes were incubated with zinc *and* insulin, there was a synergistic effect on lipogenesis.

Collipp (1984) demonstrated that a dietary zinc *deficiency* is deleterious to lipid metabolism. Markedly increased serum triglyceride concentrations were observed in Sprague Dawley rats fed a zinc-deficient diet (<3 ppm zinc) for 4 to 6 weeks, compared to rats fed a zinc supplemented diet (100 ppm zinc). To determine the cause of the elevated serum triglyceride in the zinc deficient rats, $^{14}CO_2$ production and ^{14}C-triglyceride synthesis from ^{14}C-glucose was measured in liver slices. There was greater synthesis of ^{14}C-triglyceride and less synthesis of $^{14}CO_2$ in the liver slices from the zinc deficient rats (Collip, 1984) compared to the zinc supplemented group. Although body weights and fat pad weights were not measured, the results of Collip (1984) demonstrate a potential involvement of zinc in lipid metabolism. It may be that both zinc supplementation, as demonstrated by Coulsten et al. (1980) and Chen et al. (1996), and zinc deficiency are deleterious to lipid metabolism.

The results of Coulsten et al. (1980), Chen et al. (1996), Simon (1998) and Collip (1984) denote a potential role of zinc in intermediary lipid metabolism. Although zinc supplementation may be beneficial in the treatment of hyperglycemia in type 2 diabetes, its role in adipose tissue accretion and lipogenesis needs to be further studied.

The Obese (fa/fa) Zucker Rat

Several rodent models are available for the study of diabetes and obesity. The most studied rodent models of which detailed information is available are those caused by a single gene mutation. These include the yellow, obese (ob), diabetes (db), tubby and fat (fa) mutations (Coleman, 1982). The obese fa/fa Zucker rat, first identified by Zucker and Zucker (1961) is used in this thesis project as a model of obesity and insulin

resistance. The obesity seen in this rat model is due to a single recessive autosomal gene mutation (Hainault et al., 1991).

To determine how insulin resistance develops, it is important to study the obese state before decompensation to frank diabetes (Kahn and Pederson, 1993). The obesity syndrome manifested by this rat model shares many features with human obesity. These characteristics include fat cell hypertrophy, hyperphagia, hyperlipidemia, hyperinsulinemia and normoglycemia (Hainault et al., 1991) and glucose intolerance (Ionescu et al., 1985). Thus, the obese Zucker (fa/fa) rat has been proposed to be an animal model to study the progression of type 2 diabetes (McCaleb and Sredey, 1992), although, generally, it is reported that the fa/fa rat does not develop severe hyperglycemia and thus not overt diabetes.

Studying the fa/fa rat therefore allows us to study the early stages of type 2 diabetes or the progression of the disease. In this thesis project, the Zucker rat will be the animal model to study the effects of dietary zinc manipulation on adipocyte Glut 4 expression, adiposity, hyperinsulinemia and indices of zinc status. It appears that there are no published reports of the effects of dietary zinc manipulation (zinc deficient or zinc supplementation) on the Zucker rat.

II. STUDY RATIONALE

Obesity is a key risk factor for the development of insulin resistance. Insulin resistance is the initial stage of the progression to type 2 diabetes. Research in the prevention and treatment of obesity and its associated metabolic abnormalities has focused on the role of dietary intervention. Most studies focus on the macronutrients (protein, fat and carbohydrate) and their role in disease prevention. However, several studies have identified an interrelationship among the micronutrient zinc, glucose metabolism and insulin resistance (reviewed in Literature Review). Hence, further research in the area of dietary zinc, obesity and insulin resistance warrants investigation. Although a connection between zinc and indices of diabetes has been established, the literature lacks information on the effects of dietary zinc deficiency and supplementation on the glucose transporter protein 4 (Glut 4) expression, adiposity, hyperinsulinemia and zinc status of lean and obese (fa/fa) Zucker rats. Based on a review of the literature, the hypothesis of this research project is that dietary zinc supplementation (150 ppm) would elevate the expression of Glut 4 in adipocytes, influence (elevate) body fat deposition, attenuate hyperinsulinemia and elevate zinc stores of lean and obese (fa/fa) Zucker rats, compared to rats fed zinc adequate (30 ppm zinc) and zinc deficient (5 ppm) diets.

The fa/fa Zucker rat model demonstrates obesity and insulin resistance (hyperinsulinemia) which are characteristics of human obesity. The fa/fa rat is also leptin resistant which is suggested to be a defect in human obesity as well (Caro et al., 1996). Therefore, this thesis project employs an animal model that closely mimics human obesity and insulin resistance. The fa/fa rat provides an in vivo model to assess the

effects of dietary zinc deficiency and supplementation on indices of insulin resistance and zinc status using zinc deficient (5 ppm), zinc adequate (30 ppm) and zinc supplemented (150 ppm) diets.

Understanding the progression of obesity and insulin resistance often involves studying the role of nutrition in the molecular and cellular aspects of the disease. Cellular Glut 4 is a key regulator of glucose uptake and has received ample research attention in the literature. Patients and animal models with obesity and/or type 2 diabetes may have decreased expression of adipose and/or muscle Glut 4 and the expression of this protein may be influenced by dietary factors (reviewed in Literature Review). For example, diets high in fat and ethanol influence a decrease in adipocyte Glut 4 expression in rodent models (Pederson et al., 1991; Wilkes et al., 1996). The effects of dietary zinc (deficiency or supplementation) on the expression of Glut 4 is an area that has not been researched. However, the interrelationship between zinc and insulin resistance/type 2 diabetes has been well documented in the literature. Both animal models and patients with obesity demonstrate altered zinc metabolism, as shown by lower serum and tissue zinc concentrations, and increased urinary zinc excretion (reviewed in Literature Review). Interestingly, zinc is involved with glucose metabolism and the observation of suboptimal zinc status in obesity, a risk factor for insulin resistance, raises questions regarding a possible interrelationship between altered zinc status and altered Glut 4 expression in obesity. In other words, Glut 4 and zinc are both involved with glucose homeostasis and both have been shown to be altered in obesity. Thus, the primary objective of this thesis is to investigate the relationship between dietary zinc and Glut 4 and to determine if zinc

deficiency or supplementation influences the expression of total Glut 4 in adipocytes of lean and obese (fa/fa) Zucker rats.

This study focuses on total Glut 4 expression in adipocytes. Although Glut 4 is expressed in muscle tissue as well, the interest of this project is on changes (cellular and whole body) in adiposity of the Zucker rat. Hence, isolation of epididymal adipocytes and assessment of total Glut 4 in adipocytes (but not muscle) was of particular interest. Furthermore, isolation of adipocytes allows us to study Glut 4 associated with the adipocyte cell per se. Assessing Glut 4 in whole adipose tissue would not distinguish adipocyte Glut 4 from Glut 4 that may be associated with adipose connective tissue. More importantly, the majority of the recent literature employs isolated adipocyte cells versus whole adipose tissue to assess Glut 4 expression. To enable us to compare our work to that of others, it is logical to maintain similar methodologies as those used by other authors.

Zinc is involved with the storage and release of insulin, but more importantly, zinc is associated with the action of insulin. Zinc supplementation may reduce hyperinsulinemia, as implied by studies in the hyperinsulinemic ob/ob mouse (Begin-Heick et al., 1985) and db/db mouse (Simon, 1998). The Zucker rat is an animal model of hyperinsulinemia but the effects of dietary zinc supplementation on serum insulin concentrations have not been studied in this animal. Therefore, the second objective of this thesis is to elucidate the effect of dietary zinc (deficiency or supplementation) on hyperinsulinemia of the fa/fa Zucker rat. Serum insulin in combination with serum

glucose concentrations can be collectively analyzed to investigate the degree of insulin resistance in Zucker rats after 9 weeks of dietary zinc intervention.

It is speculated that zinc supplementation may affect adipocyte Glut 4 expression and hyperinsulinemia in the Zucker rat. However, zinc supplementation may have deleterious effects on body fat accretion (adiposity). In vitro, zinc increases lipogenesis in isolated adipocytes (Coulsten et al., 1980) and at a whole-body level, zinc supplementation increases body fat deposition in obese mice (Chen et al., 1998). Considering that obesity is a major risk factor for insulin resistance and that zinc may influence (increase) body fat deposition, the effects of zinc supplementation on body fat accretion need to be identified. Hence, the third objective of this thesis is to assess changes in body fat deposition with zinc supplementation. Using body weights, epididymal and perirenal fat pad weights, and adipocyte diameters, the effects of zinc supplementation on body fat can be determined. Liver lipid analysis serves as an additional parameter to assess the connection of zinc with adipose storage.

In addition to obesity being a key risk factor of type 2 diabetes, obesity is also associated with suboptimal zinc status, as measured by lower tissue zinc stores and increases in urine zinc excretion (reviewed in Literature Review). Although the zinc status of the Zucker rat has been reported in the literature, the effects of zinc supplementation in this obese rodent model have not been studied. Thus, the fourth objective of this study is to assess the zinc status in this obese rat model and to investigate the effects of a marginal zinc deficient (5 ppm) and a zinc supplemented (150 ppm) diet for 9 weeks on zinc status in this obese rat model. Zinc status is assessed via femur and

liver zinc stores (indicators of longer-term zinc status) and serum zinc concentrations (shorter term zinc pools). Urinary zinc excretion in this study is used as an indicator of hyperzincuria allowing further insight on zinc metabolism in obesity.

In summary, the objectives of this thesis are fourfold: 1) to elucidate the effects of dietary zinc supplementation on Glut 4 expression, 2) to identify the effects of dietary zinc manipulation on hyperinsulinemia, 3) to assess body fat deposition secondary to zinc supplementation and 4) to assess the effects of zinc manipulation on tissue zinc concentrations in the Zucker rat.

The study is designed to study the effects of dietary zinc manipulation on the progression of obesity and insulin resistance. For this reason, dietary intervention was initiated at an early age (weanling, 5 weeks of age) and followed to age 15 weeks. In this manner, we can demonstrate the effects of dietary zinc on the progression of the disease. Furthermore, we felt that 15 weeks of age is an appropriate termination point given that at this age, the fa/fa rat has prominent insulin resistance, hyperinsulinemia that has reached its peak and the percentage of body lipid and fat cell size are maximum. The length of the study period is also selected based on a previous study in the db/db mouse (Simon 1998). Feeding mice a zinc supplemented diet for 6 weeks showed significant effects on zinc status and indices of diabetes. The level of zinc in the marginal zinc deficient diet (5 ppm) was based on previous studies. A marginal zinc deficiency may be achieved by feeding mice a 3 ppm zinc diet, without compromising growth of weanling mice (Simon, 1998). In weanling rats, 2.5 ppm zinc but not 5 ppm zinc, for 6 weeks results in reduced body weight gain (Gillam and Taylor, unpublished data). With a level of 5 ppm zinc

restriction, we have a further precaution that the growth of the Zucker rats will not be compromised. The zinc supplemented diet will be comprised of 150 ppm zinc, as this is sufficient to replete zinc status, without possibly causing altered copper absorption (L'Abbe and Fisher, 1984). A pair-weighed group is included in the study to control for potential weight loss in the rats fed the zinc deficient (5 ppm) diet. Rats fed a zinc deficient diet commonly experience weight loss secondary to decreased feed intake. In diabetes research, body weight is a major factor influencing insulin resistance and thus we included a pair-weighed group to control for any variables (weight loss) that would create difficulty in interpreting the results. The pair-weighed rats were provided a zinc control diet in amounts necessary to maintain the average body weights of the pair-weighed rats similar to rats fed a zinc deficient diet.

In addition to our main objectives, liver copper concentration is assessed to verify that a level of 150 ppm zinc supplementation does not induce zinc toxicity and secondary copper deficiency. Urine was collected throughout the study (0, 3, 6, 9 weeks) and analyzed for urine volume, creatinine, glucose and zinc. These measurements contribute to a more complete picture of the physiological effects of dietary zinc manipulation in this obese rat model and the progression (if any) of deteriorating renal function.

III. Methods and Materials

Animals and Diet

Male weanling (5 weeks of age) fatty (fa/fa) and lean littermate Zucker rats were purchased from Charles River Laboratories (St. Constant, PQ). Upon arrival, rats were weighed and randomly assigned to one of the eight treatment groups (shown in Table 1). At 5 weeks of age, fa/fa rats are identified by their higher body weights and relative obesity compared to the lean rats. Rats were housed in individual stainless steel cages and fed a standard lab chow diet for a one-week adaptive period, after which diet intervention was initiated. The rats were fed *ad libitum* diets low (5 ppm), adequate (30 ppm) or high (150 ppm) in zinc, based on the AIN-93G formulation (Reeves et al., 1993), for 9 weeks (diet formulations are shown in Table 2). There was also a pair-weighed group that consisted of fa/fa and lean rats fed the amount of zinc adequate diet (30 ppm) required to maintain the body weights similar to the body weights of the respective rats fed the low zinc diet. During the treatment period, the rats were maintained in a controlled environment of 21-23° C, 55% humidity and a 14-hour light, 10-hour dark cycle. The rats were provided with fresh zinc-free double deionized water, available in polypropylene bottles with stainless steel sipper tubes. Diet was provided in large glass jars and feed intake was recorded twice per week with diet spillage recorded when necessary. Body weights were recorded on a weekly basis. At weeks 0, 3, 6 and three days prior to the end of the study (~ 9 weeks), rats were weighed and then housed overnight (12 hours) in polycarbonate metabolic cages (Nalgene, Fisher Scientific) for the purpose of

urine collection. Animals were provided water *ad libitum* (but not feed) for the 12 hours to ensure that urine samples were not contaminated by zinc from the diets. Rats were fasted approximately 6 hours before termination in order to collect fasting serum samples. Animal care procedures in accordance with the Canadian Council on Animal Care (1993) were approved in a protocol submitted to the University of Manitoba Protocol Management and Review Committee.

Tissue Collection

Urine samples were collected in preweighed 50-ml polypropylene tubes that were attached to the metabolic cages. The tubes were weighed with the urine to determine urine volume and urine samples were stored at -20° C until assayed for glucose, zinc and creatinine content.

Eight animals were terminated each day at week 9 of the study, using CO_2 asphyxiation (Canadian Council on Animal Care Guidelines, 1993). Trunk blood was collected after decapitation and samples were stored on ice until centrifuged (1290 x g for 15 minutes, Beckman TJ-6 centrifuge) to obtain serum. Serum was stored in aliquots at -80° C until assayed for zinc, glucose and insulin. Several tissues were collected, weighed and analyzed as part of this study or for future analysis as part of other research. The liver, pancreas, spleen, kidney, perirenal fat pads, heart, small intestine (minus intestinal contents) and hind quarters were excised, weighed and immediately frozen in liquid nitrogen. The liver and hind quarters were stored at -20° C while all other tissues were stored at -80° C until required for analysis. The epididymal fat pad was also excised with a fresh portion used for the isolation of adipocytes.

Table 1. Dietary Treatment Groups[1]

Dietary Treatment	Fatty Rats (fa)	Lean Rats (ln)
Zinc Deficient (5 ppm)	7 faZD	7 lnZD
Control (30 ppm)	7 faZC	7 lnZC
Pair Weighed Group (30 ppm)	7 faPW	7 lnPW
Zinc Supplemented (150 ppm)	7 faZS	7 lnZS
TOTALS	**28**	**28**

[1] Abbreviations for the 8 treatment groups are shown in the body of the table. Each treatment group consisted of n=7 rats.

Table 2. Diet Formulations [1]

Ingredient	Zn Deficient Diet (5 ppm)	Zn Control Diet (30 ppm)	Zn Supplement Diet (150 ppm)
Dextrose (cerelose),[2] (g)	3619.6	3567.6	3327.6
Egg white, (g)	1275	1275	1275
Fiber (cellulose), (g)	300	300	300
Zinc-free mineral mix, (g)	210	210	210
Potassium phosphate, (g)	32.4	32.4	32.4
Vitamin mix, (g)	60	60	60
Choline, (g)	15	15	15
Biotin mix, (200 mg/kg mix)	60	60	60
Zinc premix, (5.775 g Zn carbonate/Kg mix)	8	60	300
Soybean oil,[3] (g)	420	420	420

[1] Diet ingredients were purchased from Harlan Teklad (Madison, WI) except dextrose and soybean oil. The above diet formulations were used to make 6 kg of each diet.
[2] Dextrose was purchased from Moonshiners (Winnipeg, MB)
[3] Soybean oil was purchased from Vita Health (Winnipeg, MB)

Isolation of Epididymal Adipocytes

The method used for isolation of adipocytes was based on the procedure of Rodbell (1964), Pederson et al. (1992) and Wilkes et al. (1996). The epididymal fat pads were removed, weighed and rinsed in phosphate buffered saline pH 7.4. Approximately 2-3 g of adipose tissue from each rat was used for adipocyte isolation.

The fat pad was divided into approximately 500 mg portions and each portion was carefully minced into 30-50 mg pieces using stainless steel iris scissors. The minced fat pad pieces were added to 2 ml 2.5% Albumin-Krebs Ringer Bicarbonate (albumin-KRB) buffer in 30 ml Nalgene vials. For the isolation of lean adipocytes, 1 mg/ml collagenase was added to the albumin-KRB buffer and 0.5 mg/ml of collagenase was added for the isolation of fa/fa adipocytes.

Vials were incubated at 37° C for 30 min in a shaking water bath at a speed of 50 rpm. Following the incubation with collagenase, the adipocytes were filtered through a 250-micron nylon mesh. The nylon mesh was rinsed with 8 ml albumin-KRB buffer to enhance filtration. Cells were transferred to a 15 ml falcon tube and cells were allowed to float to the top before washing.

For the removal of collagenase, cells were washed 3 times with buffer, allowing the cells to completely float to the top between washes. Cells were resuspended in 10 ml albumin-buffer and an aliquot of cells was used to determine diameter by image analysis. Due to the fragility of the larger fa/fa

adipocytes, cells were easily ruptured/lysed during repeated washing and aliquoting. Therefore, an accurate cell count was not obtained for the fa/fa rats and thus adipocyte number per fat pad (both lean and fa/fa) is not reported in this thesis.

For the removal of albumin, cells were washed an additional 3 times (5 ml buffer per wash) with albumin-free buffer containing protease inhibitors (1 ng/ml each of aprotinin, leupeptin and phenylmethylsulfonyl fluoride). The final supernatant fraction was removed and the cell pellet was frozen at -80° C.

The lean cells were washed with albumin-KRB buffer to remove the collagenase, followed by washing with albumin-free phosphate buffered saline, pH 7.4, for the removal of albumin. The fa/fa adipocytes were washed with TES buffer (20 mM Tris-HCl, 1mM EDTA, 255 sucrose, pH 7.4) for the removal of collagenase and albumin. As mentioned, the fa/fa adipocytes were more fragile and therefore prone to lysing during the washing procedure. The TES buffer provided a more dense solution to aid in suspending the larger fa/fa adipocytes, compared to the water-based PBS buffer. The use of the TES buffer in isolating fa/fa adipocytes is suggested by Pederson et al. (1992) and does not alter Glut 4 content of the cells.

Protein Assay for the Determination of Total Adipocyte Protein

The Bicinchoninic Acid Protein Assay Kit (Sigma Procedure #TPRO-562) was used to determine of total protein concentration in adipocytes. This procedure is based on the principle that proteins reduce alkaline Cu (II) to Cu (I) in a concentration dependent manner. The bicinchoninic acid is a highly specific

chromogenic reagent for Cu (I), ultimately forming a purple complex with an absorbance maximum at 562 nm. The absorbance at 562 nm is directly proportional to the protein concentration in the given sample.

a) Reagents and Equipment

- Bicinchoninic Acid (BCA) Solution
- Copper (II) Sulfate Pentahydrate 4% Solution
- Protein Standard Solution [Sigma, Bovine Serum Albumin, 1 mg/ml]
- 96-well Polystyrene Microtiter Plate Wells [#25880-96, Corning Glass Works, Corning, NY]
- SpectroMax Microtiter Plate Reader [Molecular Devices Corporation, Sunnyvale, CA]

b) Procedure

Frozen adipocyte cell pellets were thawed at room temperature, sonicated (10 minutes) and centrifuged at 500 x g (5 minutes) at 4°C. The bottom protein layer was carefully removed using a syringe and transferred to 1.5 ml microcentrifuge tubes. The protein aliquots were kept on ice during protein concentration analysis.

Bovine serum albumin was used to establish a standard curve. Samples, standards (diluted in albumin-free phosphate buffered saline) and buffer blanks of 10 *u*l were pipetted in triplicate into 96-well plates. Two hundred *u*l BCA reagent [1 part Copper (II) sulfate solution: 50 parts BCA solution, prepared fresh daily] was added to the wells using a multi-channel pipette. Samples on the microplate were shaken for 5 seconds by the plate reader and allowed to incubate for 30

minutes at 37° C. Absorbencies were read at 562 nm and the software program calculated unknown sample concentrations (mg/ml). If unknown samples were above the standard curve, samples were appropriately diluted to fit within the range and the assay was repeated.

c) Calculation

$$\text{Protein Concentration (mg/ml)} = \frac{\text{Concentration of Standard} \times [A]\text{Sample}}{[A]\text{ Standard}} \times \text{Dilution}$$

Where [A]=the absorbance reading

Following the determination of protein concentration, the protein samples were frozen in approximately 55 μg protein aliquots at -80° C in preparation for analysis of total Glut 4 expression.

Western Immunoblotting of Glut 4 in Adipocytes

This procedure was used to determine the expression of total Glut 4 in adipocytes isolated from epididymal fat pads of lean and fa/fa rats.

a) Solutions and Materials

- 10% Sodium Dodecyl Sulfate (SDS) Separating Gel [4.0 ml H_2O, 3.3 ml 30% Acrylamide mix, 2.5 ml 1.5 M Tris (pH 8.8), 0.1 ml 10% SDS, 0.1 ml 10% APS (made fresh daily), 0.004 ml TEMED]
- 5% SDS Stacking Gel [1.4 ml H_2O, 0.33 ml 30% Acrylamide mix, 0.25 ml 1.0 M Tris (pH 6.8), 0.02 ml 10% SDS, 0.02 ml 10% APS (made fresh daily), 0.002 ml TEMED]
- Electrode Buffer [3 g/L Tris, 14.4 g/L glycine, 1 g/L SDS]

- Transfer Buffer [3.03 g/L Tris, 14.4 g/L glycine, 200 ml/L methanol, pH 8.3]
- Tris-Buffered Saline (TBS) [6.05 g/L Tris, 8.76 g/L NaCl, pH 7.5]
- TBS-Tween [999 ml TBS (pH 7.5), 1 ml Tween-20]
- 6X SDS Sample Buffer [7 ml 4x Tris-HCl/SDS (pH 6.8), 3.0 ml glycerol, 1 g SDS, 0.93 g Dithiothreitol (DTT), 1.2 mg bromophenol blue]
- 4X Tris-HCl/SDS, pH 8.8 [Dissolve 6.05 g Tris in 40 ml H_2O. Adjust to pH 6.8 using 1N HCl. Add H_2O to 100 ml total volume. Add 0.4 g SDS]
- Skim milk solution (5% and 2% w/v) [skim milk powder dissolved in TBS-Tween]
- Glut 4 Antibody, goat polyclonal IgG (C-20) [Santa Cruz Biotechnology, Santa Cruz, California; catalogue #sc1608]
- Anti-goat IgG-Horse Radish Peroxidase (HRP) [Santa Cruz Biotechnology, Catalogue #sc2243]
- Prestained Protein Molecular Weight Standards [GibcoBRL, Burlington Ontario; 14,300-200,000 molecular weight range]
- Nitrocellulose Transfer Membrane, 0.45 micron [BioRad Laboratories, Mississauga, Ontario]
- ECL Plus Western Blotting Detection Reagents [Amersham Pharmacia Biotech, Baie d'Urfe, Quebec, Canada]
- Western Blocking Reagent [Boehringer Mannheim, Laval, Quebec; diluted to 0.5% using TBS]
- 10 well comb and 1.5 mm Spacers [BioRad]

b) Procedure

SDS-polyacrylamide gels were prepared on a BioRad Mini-PROTEAN™ II Cell Apparatus. The separating gel was poured using a pasteur pipette, covered with water-saturated isobutanol to produce an even surface and allowed to polymerize for 30 minutes. Following polymerization, the isobutanol was poured off, the top of the separating gel was rinsed with distilled water and any residual water was absorbed with gel blotting paper. The stacking gel was poured on top of the separating gel and a 10 well comb was inserted. Polymerization of the stacking gel was allowed for 15-30 minutes after which time the comb was removed.

Fifty micrograms of adipocyte protein sample was solubilized by the addition of 6X SDS sample buffer or 4X SDS sample buffer [prepared by diluting 6X SDS sample buffer with H_2O]. The use of 6x SDS sample buffer was needed for samples that were too dilute and hence the addition of 4x sample buffer would result in volumes greater than the capacity of the wells (approximately 97 ul). The final volume of both the protein sample and SDS sample buffer did not exceed 90 *u*l. An adipocyte protein sample was selected as the internal control and was included on all western blots.

The sample was gently vortexed, centrifuged for 4 seconds at 6800 rpm and boiled for 4 minutes. Proteins contained within the sample were then separated according to molecular weight by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Electrophoresis took place using 800 ml electrode buffer at 170 V [Power Pac 200] for 1 hour.

One piece of nitrocellulose membrane and 6 pieces of gel blotting paper were cut to the size of the gel and soaked in transfer buffer for 30 minutes. The gel was then rinsed briefly in transfer buffer and assembled in a BioRad Mini Trans-Blot Cell apparatus between a triple layer of gel blotting paper. Transfer of proteins from the gel to the nitrocellulose was completed after 90 minutes at 100 V on ice.

The membrane was soaked in 15 ml 5% skim milk solution with gentle shaking (50 rpm) for 1 hour at room temperature. The membrane was then briefly rinsed with TBS-Tween. The Glut 4 antibody was diluted 1:100 using 0.5% western blocking reagent and incubated with the membrane overnight at 4° C with constant shaking in a petri dish. Following the overnight incubation with the Glut 4 antibody, the membrane was washed 3 times (10 minutes each wash) using 10 ml TBS-Tween for each wash. The membrane was then incubated with a 1:1,000 dilution anti-goat antibody conjugated to HRP in 15 ml 2% skim milk solution (dilutions were made immediately prior to use) for 1 hour with shaking (50 rpm) at room temperature. The membrane was washed 3 times using 10 ml TBS-Tween and then placed on a piece of plastic saran wrap. An ECL Plus chemiluminescence kit was used to detect the bound secondary antibody. The two solutions provided in the kit were mixed prior to use at a ratio of 1.5 ml Solution A: 37.5 ul solution B per membrane. The ECL solution was added to the membrane using a pasteur pipette, ensuring that the entire membrane was exposed to the solution, and incubated at room temperature for 1 minute. The membrane was immediately transferred to a dry piece of plastic wrap, covered, exposed to

film for various times (10-20 minutes) and developed using GBX Kodak Developer (Don's Photo, Winnipeg, MB).

In order to analyze the expression of total Glut 4, bands were scanned by image analysis (Hewlett Packard ScanJet 4c) and assigned arbitrary units which represent band area combined with gray intensity in comparison to a control sample. The band area and gray intensity of the bands were analyzed using computer software (Scion Image 2.0 Software for Microcomputers). For each blot, arbitrary units for the treatment groups were normalized to the internal control sample.

Insulin Assay

Insulin in the serum was assayed using a sensitive rat insulin radioimmunoassay kit (#SRI-13K, Linco Research Inc., St Charles, MO). The procedure follows the basic principle of radioimmunoassay where there is a competition between radioactive and non-radioactive antigen for a fixed number of antibody binding sites. The amount of ^{125}I-insulin bound is inversely proportional to the concentration unlabelled insulin.

a) Reagents

- Assay Buffer: 0.05 M Phophosaline (pH 7.4) containing:0.025 M EDTA, 0.1% Sodium Azide, 1% RIA grade, Bovine Serum Albumin
- Sensitive Rat Insulin Antibody
- ^{125}I-Insulin label (<3 μCi/27 ml) hydrated in assay buffer containing Normal Guinea Pig IgG as carrier.
- Insulin standards (0.02-1.0 ng/ml) and Quality controls
- Precipitating reagent

b) Procedure

On day 1, serum insulin samples were diluted in Assay Buffer (5 fold for lean rats and 200 fold for fa/fa rats) to be within range of the standard curve. Assay buffer was pipetted into Borosilicate glass (12 x 75 mm) tubes: 300 *u*l into 2 non-specific binding tubes, 200 *u*l into 2 total binding reference tubes, and 100 *u*l into tubes for standards, controls and unknowns. One hundred *u*l of standards, quality controls, or diluted sample were pipetted in duplicate into appropriate tubes. One hundred *u*l rat insulin antibody was pipetted into all tubes except total count and non-specific binding tubes. Tubes were vortexed, covered with parafilm, stored in a styrofoam test tube rack in a sealed plastic container, and incubated overnight at 4° C. The following day, 100 *u*l ^{125}I-insulin was pipetted into all tubes. Tubes were vortexed, covered with parafilm, stored in a styrofoam test tube rack in a sealed plastic container, and incubated overnight at 4° C. On the third day, 1.0 ml precipitating reagent was added to all tubes except total count tubes. Tubes with the precipitating reagent were vortexed, incubated for 20

minutes at 4° C, and centrifuged for 30 minutes at approximately 2000 x g to achieve a firm pellet. The supernatant fraction was decanted and tubes were held inverted for 1 minute for complete blotting of all liquid. Remaining pellets were counted for ^{125}I in a gamma counter (Beckman Gamma 8000, Scientific Instruments, Irvine, CA).

c) Calculation

- Total count tubes = tubes with ^{125}I only
- Non-specific binding tubes = tubes with ^{125}I and precipitating agent (no insulin antibody, sample or standard)
- Total binding reference tubes = tubes with all reagents except sample or standard
- Non-specific binding count was subtracted from all tubes except total counts.

$$\text{Percentage of maximum binding } (\%B/B_o) = \frac{\text{Sample or Standard cpm}}{\text{Total Binding Reference cpm}} \times 100$$

A reference curve (based on the standards) was developed using Prism computer software program to calculate the equation of the line and to solve for unknowns. Insulin concentrations (ng/ml) for unknown samples and quality controls were then determined by interpolation on this standard reference curve.

Serum and Urine Glucose

The procedure to measure serum and urine glucose concentration is based on 2 coupled enzymatic reactions using glucose oxidase (Reaction #1) and peroxidase enzyme (Reaction #2).

Reaction #1: Glucose + H_2O + O_2→Gluconic Acid + H_2O_2

Reaction #2: 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 original glucose concentration in the serum and urine samples.

a) Reagents and Materials

- Glucose Assay Kit [Sigma Diagnostics, Procedure #510]
- PGO Enzymes [Catalog #510-6]
- Enzyme Solution [Prepared by adding contents of 1 capsule of PGO enzymes to 100 ml distilled water in an amber bottle]
- o-Dianisidine Dihydrochloride [Catalog #510-50]
- Color Reagent Solution [Prepared by reconstitution one vial of o-Dianisidine Dihydrochloride with 20 ml water]
- Combined Enzyme-Color Reagent Solution [Prepared by combining 100 ml enzyme solution and 1.6 ml Color Reagent Solution]
- Glucose Standard Solution [Catalog #635-100, 100 mg/dl (5.56 mmol/L) in 0.1% benzoic acid solution]
- 96-well Polystyrene Microtiter Plate [#25880-96, Corning Glass Works, Corning, NY]
- SpectroMax Microtiter Plate Reader [Molecular Devices Corporation, Sunnyvale, CA]

b) Procedure

Glucose standard was diluted and used to establish the standard curve (0-10 mg/dl). Urine and serum samples were diluted 10-fold to determine concentration based on the standard curve followed by further dilution (20-200-fold) if required. Samples, standards and buffer blanks of 20 *u*l were pipetted in triplicate into 96-well plates. Two-hundred *u*l combined enzyme-color reagent solution was pipetted into the wells using a multi-channel pipette. The plate was placed in the spectrophotometer drawer, mixed and allowed to incubate at 37°C for 30 minutes. Absorbencies were read at 450 nm and concentration of glucose in the samples was calculated based on the standard curve.

c) Calculation

$$\text{Glucose Concentration (mg/dl)} = \frac{\text{Concentration of Standard} \times [A]\text{Sample}}{[A]\text{ Standard}} \times \text{Dilution}$$

Where [A]=absorbance

Conversion of Units: mMol/L = mg/dl x 0.0555

Urine Creatinine

Creatinine is a urinary excretion product that is proportional to body muscle mass (Bowers and Wong, 1980). Since creatinine excretion from the body is relatively constant, it was used in this study as a basis for expressing urinary concentration of glucose and zinc and is referred to as "corrected urinary glucose or zinc concentrations." The corrected urinary values thus take into account differences in urine volume as reflected by creatinine excretion. A colorimetric

creatinine assay kit (Sigma Diagnostics, Procedure #555) was adapted to a microplate method and was used to determine the creatinine concentration in urine collected from lean and fa/fa rats. This procedure employs the principle that upon contact with alkaline picrate solution, a sample will form an orange/yellow colour. This colour is derived solely from creatinine and is destroyed by the addition of an acidic pH solution. Therefore, the difference in colour intensity, measured at 500 nm before and after acidification, is proportional to creatinine concentration.

a) Reagents

- Creatinine Color Reagent [Catalog #555-1, 0.6% Picric Acid, sodium borate and surfactant]
- Acid Reagent [Catalog #555-2, mixture of sulfuric acid and acetic acid]
- Creatinine Standard [Catalog #925-3, Creatinine, 3.0 mg/dl (265 *u*mol/L) and 15 mg/dl (1325 *u*mol/L) in 0.02 N HCl]
- Sodium Hydroxide Solution [Catalog #930-55, 1.0 N]
- Alkaline Picrate Solution [Prepared by mixing 5 volumes of Creatinine Color Reagent with 1 volume of Sodium Hydroxide Solution]
- 96-well Polystyrene Microtiter Plate [#25880-96, Corning Glass Works, Corning, NY]
- SpectroMax Microtiter Plate Reader [Molecular Devices Corporation, Sunnyvale, CA]

b) Procedure

Urine samples collected at baseline, 3, 6 and 9 weeks were diluted 10 - 20 fold times using distilled water. Creatinine standards were used to develop a standard curve (0-15 mg/dl) from which unknown concentrations were determined. The alkaline picrate reagent was prepared fresh daily and stored in the dark at room temperature.

Samples, standards and buffer blanks of 20 *u*l were pipetted in triplicate into 96-well plates. Twenty *u*l alkaline picrate reagent was pipetted into the wells using a multi-channel pipette. The plate was placed in the spectrophotometer drawer, mixed and allowed to incubate for 10 minutes before reading the initial absorbance at 500 nm. The plate was removed from the drawer, 7 *u*l acid reagent was pipetted into the wells using a multi-channel pipette and the plate was mixed in the spectrophotometer. The acid reagent was allowed to incubate for 5 minutes and then a second reading was recorded. Absorbencies were read at 500 nm and the software program calculated unknown sample concentrations (mg/dl). If unknown samples were above the standard curve, samples were appropriately diluted to fit within the range and the assay was repeated.

c) Calculation

$$\text{Creatinine Concentration (mg/dl)} = \frac{\text{Concentration of Standard X [A] Sample}}{\text{[A] Standard (3 mg/dl)}} \text{ (X 3) (X Dilution)}$$

Where [A]=absorbance

Liver Lipid

The method of Folch (1956) was used to quantify total lipid content in the liver. The method utilizes specific ratios of chloroform, methanol and water to extract the lipid fraction in a two day procedure.

a) Procedure

Livers were thawed, cut into 1.0 g portions and placed in 22 ml of a 2:1 (by volume) chloroform/methanol solution. Each liver was homogenized twice at 20 second intervals using setting #4 on a Polytron Homogenizer (model #PT 1020 3500, 115 volt, Brickmann Instruments, Rexdale, ON). The homogenate was filtered (#1 Whatman filter) and the volume of eluate collected in a 25 ml graduated cylinder was recorded. Twenty percent of this volume was added as water, shaken gently by inversion with venting, covered and allowed to separate overnight.

The following day, the top methanol layer and a protein disc layer were removed using an aspirator. Ten milliliters of the bottom chloroform layer were carefully removed and placed in a 25-ml glass vial that was previously dessicator dried and weighed. The chloroform was evaporated from the vial using a heated water bath (OA-SYS heating system, Organomation Associates, Berlin, MA) with a stream of nitrogen air (0.7 Kg/cm^2) for one hour. The lipid-containing vials were allowed to cool in the dessiccator for a minimum of one hour. They were then weighed for lipid content.

b) Calculation

Liver lipid (g) =

$$\frac{\text{(Dried vial \& lipid) - dried vial alone}}{\text{Chloroform volume removed (10 ml)}} \times \frac{\text{Volume of initial chloroform layer}}{\text{Amount of liver tissue used (1 g)}}$$

Zinc Analysis

Femurs, livers, urine, serum and diets were analyzed for zinc using Atomic Absorption Spectrophotometry. Serum and urine samples were subjected to a direct analysis at a 10 and 4 fold dilution (respectively) in double deionized water, while femurs, livers and diets required acid digestion prior to zinc analysis.

The acid digestion of tissues and diets was based on the technique of Clegg et al. (1981). Hind quarters were thawed, the femurs were carefully scraped of all musculature using a scalpel blade and femur lengths (using 30 cm ruler) and wet weights were recorded. Similarly, livers were thawed and cut into approximately 500 mg portions. Approximately 0.1 g of each diet was used for zinc analysis in addition to 150 mg of a bovine liver reference, which was used as a quality control (ref.#1577b, U.S. Department of Commerce, National Institute of Standards and Technology, Gaithersburg, MD).

Femurs and livers were dried for 48 hours in a drying oven set at 85° C. After 48 hours, dry weights were recorded. Each femur, liver and diet/reference sample was placed in a dry test tube that was previously washed with 30% nitric acid. A 1 ml aliquot of 70% nitric acid, trace element grade (VWR Canlab, Mississauga, ON) was added to each sample and blanks, and all test tubes were covered with an acid washed glass marble. Digestion proceeded for 2 hours at

room temperature followed by 48 hours with heating at 85° C using a dry bath heater.

a) Dilution of Samples for Zinc Analysis

All dilutions were prepared using double deionized water and acid-washed volumetric flasks.

- ZD femur samples were diluted to 100 ml.
- ZC and ZS femur samples were diluted to 250 ml.
- Liver samples were diluted to 10 ml and further diluted 4 fold (total dilution of 40).
- ZD and ZC diet samples were diluted to 10 ml.
- ZS diet samples were diluted to 25 ml.

Samples were analyzed using an atomic absorption spectrophotmeter (Varian Spectra AA-30 Spectrophotometer, Georgetown, ON) and zinc standards (0.1-1 ppm) were prepared from a zinc atomic absorption standard (1000 ppm, #H595-01 Mallinckrodt, Paris, Kentucky).

b) Calculations

For femur and diet zinc

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

For serum and urine zinc

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

Femur Calcium and Phosphorus, and Liver Copper

Mineral content of the femur was further analyzed for calcium and phosphorous concentrations using the same dilution factors as those used for zinc analysis. Femur calcium and phosphorous samples were analyzed in triplicate using an Emission Spectrometer (Varian Liberty 200 ICP, Varian Canada, Georgetown, ON). Liver copper samples were analyzed in triplicate using a 40 fold dilution factor for all samples.

a) Calculations

For femur calcium and phosphorous

$$\text{Calcium or Phosphorus } (\mu g/g \text{ dry weight}) = \frac{\text{Sample Ca or P concentration X dilution factor}}{\text{Dry weight of sample}}$$

For liver copper

$$\text{Copper } (\mu g/g \text{ dry weight}) = \frac{\text{Sample copper concentration X dilution factor}}{\text{Dry weight of sample}}$$

Statistical Analysis

Differences between dietary treatment groups were analyzed by two-way ANOVA (SAS 6.04, SAS Institute, Cary, NC). Main effects were genotype, zinc, and the interaction of genotype x zinc. Duncan's multiple range test was used as a stringent statistical method to determine significant differences between means. Correlation analysis on serum zinc, liver zinc and femur zinc data was performed using the Pearson's Product Correlation Coefficient. Arbitrary units for Glut 4 were log transformed for ANOVA and means testing are reported as non transformed data. Repeated measures ANOVA was used to determine differences

between time points for body weight and urine data. In all analyses, differences were accepted as significant at $p<0.05$. Each table or figure in the results section shows the mean ± standard error of the mean (SEM) for n=7 samples unless otherwise indicated in the legend.

IV. Results

There were several research questions investigated in this thesis project and the results can be divided into 1) results/effects due to *genotype* and 2) effects due to *dietary zinc*. The genotype effects refer to significant differences observed in our investigations that are due solely to the genetic differences between the obese (fa/fa) and their lean littermates. The dietary zinc effects refer to significant differences observed as a result of the dietary manipulation of zinc. The effects due to genotypic, dietary zinc and the interaction of genotype and dietary zinc will be reported in the results. All data is presented in tabular form. For data in figures, the means and standard errors of the mean are also presented in tabular form within the results section. Main effects for genotype, dietary zinc and the interaction of genotype and dietary zinc are reported in Appendixes A through E.

Feed Intake

Feed intake was recorded weekly and the total feed intake per dietary treatment group for the duration of the 9 week experiment is shown in Table 3. The feed intakes of the faPW and lnPW groups were similar to the amounts of diet consumed by the respective ZD groups and thus were not included in the analysis. The purpose of including a pair-weighed group in this experiment (as discussed in the Study Rationale) was to control for potential changes (decreases) in body weight of the ZD rats that would have ultimately confounded our results. Thus we included a PW group for each genotype and provided daily amounts of zinc control diet (30 ppm) that matched the feed intake of the ZD rats. The lean ZD and fa/fa ZD rats consumed 17-25 grams and 28-35 grams diet (5 ppm zinc) per day, respectively. Therefore, the lean PW and fa/fa PW rats were

provided approximately 20 grams and 32 grams of ZC (30 ppm) diet per day, respectively, in order to maintain the PW body weights similar to their respective ZD groups. An interesting observation was noted on the meal pattern/behavior of the pair-weighed rats. Firstly, rats in the ZD, ZC and ZS dietary treatment groups were provided an "unlimited" amount of diet. That is, feed jars were filled twice per week and rats "nibbled" throughout the day and night. On the contrary, the PW rats were provided specific amounts of diet *once per day*. After routine observations during the 9 week intervention period, we observed that the PW rats would consume all the diet provided within 3 hours and were then essentially "fasting" until the next day's feed was provided. This pattern of eating is similar to the feeding patterns of meal-fed rats.

There was a significant effect of genotype on the total feed intake of fa/fa and lean rats throughout the 9 weeks dietary intervention period (Appendix A). The fa/fa rats consumed more diet than the lean rats (1950 ± 41 vs. 1437 ± 51 grams, respectively) but there were no differences in total feed intake among the dietary treatment groups as shown in Table 3 and Figure 1.

Body Weight and Body Fat

There was a significant main effect of genotype on body weight and body fat after 9 weeks of dietary zinc intervention (Appendix A). The fa/fa rats had significantly heavier body weights compared to lean rats (611 ± 9 vs. 366 ± 6 grams, respectively) in addition to heavier epididymal fat pads (18.4 ± 0.7 vs. 6.4 ± 0.3 grams, respectively) and heavier perirenal fat pads (27.2 ± 0.9 vs. 6.6 ± 0.3 grams, respectively). Therefore, at the end of the experiment, fa/fa rats had body weights that were 40% heavier, and the epididymal and perirenal fat pads were 65.3% and 75.9% heavier, respectively, compared

to the lean rats. Table 4 shows the results for body weight and body fat after 9 weeks of dietary treatment among the 8 treatment groups. There were no differences in final body weights due to dietary zinc, and the body weights of the PW rats were similar to the ZD rats within genotype groups. Table 5 and Table 6 shows body weights and body weight gain among dietary treatment groups during the 9 week experiment. At weeks 0, 3, 6 and 9, the fa/fa rats had significantly heavier body weights compared to lean rats and there were no differences within genotype groups at all time points. Further analysis of the body weight data indicates that the increases in body weight observed every 3 weeks were significantly higher than the previous time point for both genotypes and for all treatment groups (Appendix F). The fa/fa rats gained significantly more weight (on a weekly basis) compared to lean rats. When dietary treatment groups are compared, there were no differences in weekly weight gain among the lean rats at all time points (Table 6). However, there were differences observed in the fa/fa rats at weeks 3 and 5. Between week 2 and week 3, the faZC rats gained significantly more weight than the faZD rats (Table 6). At 5 weeks, the faPW rats gained significantly less weight compared to the faZD, faZC and faZS groups. In summary, the fa/fa rats weighed significantly more and gained significantly more weight compared to the lean rats at all time points measured.

Fat pad weights were recorded as a measurement of obesity. Similar to body weight, there was a significant effect of genotype but not dietary zinc on epididymal and perirenal fat pad weight or their combined total fat pad weight. The relative weights of both fat pads were calculated to take into account differences in body weights. Relative fat pad weight (epididymal, perirenal and total) was significantly higher in the fa/fa genotype. There was a significant genotype x zinc interaction for the relative epididymal

fat pad weight, but not the perirenal fat weight (Appendix A). The relative epididymal fat weight was significantly greater (16.7%) in the faPW group compared to the faZS group. There were no significant differences observed within the lean rats, although there was a trend towards a greater relative epididymal fat weight in the lnZS group. The relative total fat weight (sum of epididymal and perirenal fat pad weight expressed as grams per 100 grams body weight) was greater in the faPW group compared to the faZS group and there were no differences among the lean rats. Therefore, the pair weighed fa/fa rats had larger relative fat pad weights indicating a possible relationship between feeding patterns, genotype and fat deposition in epididymal and perirenal fat pads.

Adipocyte Diameter

Adipocyte diameters were used as another indicator for changes in body fat between genotypes and among dietary treatment groups. There was a significant effect of genotype on the diameters of the adipocytes (Appendix A). The fa/fa adipocytes had diameters that were 19.7% larger than lean rats (105 ± 2 vs. 84 ± 2 μm, respectively). There was no significant main effect of zinc on the adipocyte diameters (Appendix A) and as reported in Table 4, there were no differences in adipocyte diameters within the fa/fa or lean dietary treatment groups.

Organ Weight: Kidney, Small Intestine, Pancreas and Spleen

There was a significant genotype effect on kidney weights (Appendix B). The kidney weights were 26.8% heavier in the fa/fa rats compared to the lean rats (4.1 ± 0.1 vs. 3.0 ± 0.0 grams, respectively) and there were no differences in kidney weights due to dietary treatment as shown in Table 7.

Table 3. Effects of dietary zinc on total feed intake of obese (fa/fa) and lean Zucker rats after 9 weeks of dietary treatment[1]

	Dietary Treatment Groups[2]					
	fa/fa			lean		
	faZD	**faZC**	**faZS**	**lnZD**	**lnZC**	**lnZS**
Total Feed Intake (g)	1936[a] ±64	1932[a] ±85	1983[a] ±74	1406[b] ±96	1425[b] ±96	1479[b] ±87

[1] Values represent the mean ± SEM for n=7 rats. Different superscript letters indicate significant differences between means as determined by Duncan's multiple range test.

[2] fa/fa=obese Zucker rat, lean=lean Zucker rat, faZD=fa/fa zinc deficient diet, faZC=fa/fa zinc control diet, faZS=fa/fa zinc supplemented diet, lnZD=lean zinc deficient diet, lnZC=lean zinc control diet and lnZS=lean zinc supplemented diet.

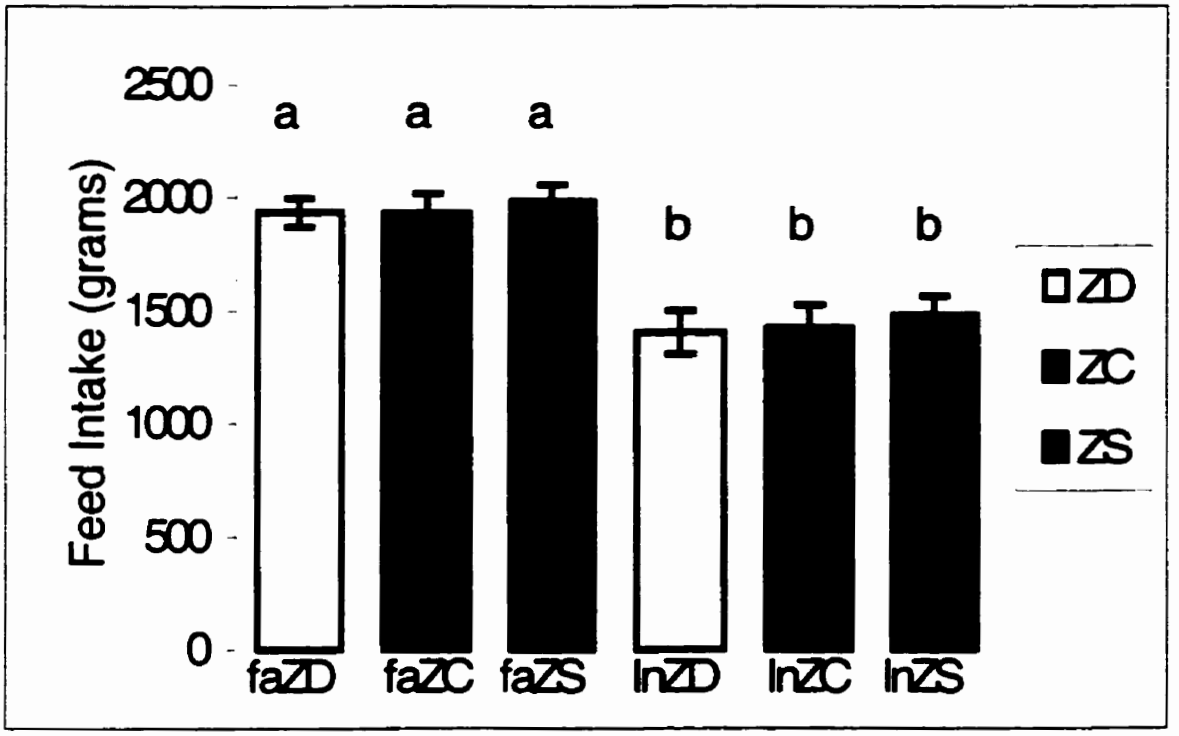


Figure 1. The effects of dietary zinc on total feed intake of obese (fa/fa) and lean Zucker rats after 9 weeks of dietary treatment. Treatment groups were faZD=fa/fa zinc deficient diet, faZC=fa/fa zinc control diet, faZS=fa/fa zinc supplemented diet, lnZD=lean zinc deficient diet, lnZC=lean zinc control diet and lnZS=lean zinc supplemented diet. Each bar represents the mean feed intake ± SEM for n=7 rats. Different lowercase letters indicate significant differences between means as determined by Duncan's multiple range test.

Relative kidney weights were calculated to take into account differences in body weight and the results demonstrated a significant main effect of genotype (Appendix B). Interestingly, the fa/fa rats had a significantly lower (18.3%) *relative* kidney weight compared to lean rats (0.67 ± 0.01 vs. 0.82 ± 0.01 grams, respectively). In terms of dietary treatment, the relative kidney weight was greater in the faPW group compared to the faZC group and no differences were observed among the lean rats (Table 7).

A significant genotype effect was observed on small intestine weight (Appendix B). The fa/fa rats had heavier small intestine weights compared to lean rats (8.2 ± 0.2 vs. 5.8 ± 0.1 grams, respectively) but no differences were observed among the fa/fa or lean dietary treatment groups, as shown in Table 7. However, calculation of the relative small intestine weight showed that the *relative* small intestine weight was significantly lower in the fa/fa rats compared to lean rats (1.3 ± 0.0 vs. 1.6 ± 0.0 grams, respectively). Dietary zinc did not significantly affect the relative small intestine weights among genotype groups. In addition to small intestine weight, small intestine length was also recorded and analysis of the results showed a significant main effect of genotype (Appendix B). The fa/fa rats had a longer small intestine compared to the lean rats (89.2 ± 2.7 vs. 76.3 ± 1.9 cm, respectively). In terms of diet, the lnZC small intestine length was significantly shorter than the faZD, faZC and faZS groups.

The results for the effect of dietary zinc on pancreas and spleen weights are shown in Table 8. There was a significant genotype effect on pancreas weight (Appendix B). The fa/fa rats had a 23.5% lower pancreas weight compared to the lean rats (1.3 ± 0.1 vs. 1.7 ± 0.1 grams, respectively). A comparison among dietary treatment groups showed

Table 4. Effects of dietary zinc on body weight, body fat measurements and adipocyte diameters of obese (fa/fa) and lean Zucker rats after 9 weeks of dietary treatment[1]

	Dietary Treatment Groups[2]							
	fa/fa				lean			
	faZD	faZC	faPW	faZS	lnZD	lnZC	lnPW	lnZS
Body Weight (g)	608[a] ±22	620[a] ±18	599[a] ±16	619[a] ±19	350[b] ±11	368[b] ±13	359[b] ±12	387[b] ±10
Epididymal Fat Pad Weight (g)	18.6[a] ±1.5	18.7[a] ±1.2	19.4[a] ±1.5	16.8[a] ±1.2	5.66[b] ±0.53	6.23[b] ±0.26	6.16[b] ±0.59	7.47[b] ±0.69
Perirenal Fat Pad Weight (g)	26.8[a] ±1.9	26.4[a] ±1.6	28.2[a] ±2.1	27.3[a] ±1.6	5.87[b] ±0.66	5.94[b] ±0.30	6.63[b] ±0.63	7.74[b] ±0.78
Total Fat Pad Weight (g)	45.4[a] ±2.9	45.0[a] ±2.4	47.6[a] ±3.2	44.2[a] ±2.5	11.5[b] ±1.2	12.2[b] ±0.6	12.8[b] ±1.1	15.2[b] ±0.0
Relative Epididymal Fat Weight (g/100 g body weight)	3.05[ab] ±0.20	3.01[ab] ±0.15	3.24[a] ±0.20	2.70[b] ±0.11	1.60[c] ±0.12	1.69[c] ±0.03	1.70[c] ±0.12	1.91[c] ±0.16
Relative Perirenal Fat Weight (g/100 g body weight)	4.37[a] ±0.17	4.23[a] ±0.17	4.67[a] ±0.25	4.41[a] ±0.15	1.66[b] ±0.15	1.62[b] ±0.05	1.84[b] ±0.16	1.98[b] ±0.16
Relative Total Fat Weight (g/100 g body weight)	7.43[ab] ±0.25	7.25[ab] ±0.23	7.91[a] ±0.35	7.11[b] ±0.20	3.26[c] ±0.26	3.31[c] ±0.07	3.54[c] ±0.23	3.90[c] ±0.30
Adipocyte Diameter (um)	107[a] ±7 (n=4)	98.8[ab] ±6.1 (n=5)	104[a] ±2 (n=5)	111[a] ±4 (n=5)	84.9[c] ±5.4 (n=5)	81.8[c] ±3.1 (n=6)	83.9[c] ±5.1 (n=5)	86.9[bc] ±2.8 (n=5)

[1] Values represent the mean ± SEM for n = 7 rats except for adipocyte diameter as indicated in the table. Different superscript letters indicate significant differences between means as determined by Duncan's multiple range test.

[2] fa/fa=obese Zucker rat, lean=lean Zucker rat, faZD=fa/fa zinc deficient diet, faZC=fa/fa zinc control diet, faPW=fa/fa pair-weighed, faZS=fa/fa zinc supplemented diet, lnZD=lean zinc deficient diet, lnZC=lean zinc control diet, lnPW=lean pair-weighed and lnZS=lean zinc supplemented diet.

Table 5. Effects of dietary zinc on body weight of obese (fa/fa) and lean Zucker rats at 0, 3, 6 and 9 weeks of dietary treatment[1]

	Dietary Treatment Groups[2]							
	fa/fa				lean			
	faZD	faZC	faPW	faZS	lnZD	lnZC	lnPW	lnZS
Week 0 Body weight (g)	192[a] ±11	198[a] ±11	193[a] ±12	196[a] ±10	121[b] ±5	122[b] ±6	121[b] ±4	126[b] ±4
Week 3 Body weight (g)	396[a] ±13	407[a] ±11	406[a] ±15	408[a] ±12	236[b] ±7	255[b] ±5	253[b] ±6	264[b] ±6
Week 6 Body weight (g)	535[a] ±18	555[a] ±15	529[a] ±16	550[a] ±16	305[b] ±8	316[b] ±9	310[b] ±8	335[b] ±10
Week 9 Body Weight (g)	608[a] ±22	620[a] ±18	599[a] ±15	619[a] ±19	350[b] ±11	368[b] ±13	359[b] ±11	387[b] ±10

[1] Values represent the mean ± SEM for n=7 rats. Different superscript letters indicate significant differences between means within a given week as determined by Duncan's multiple range test.

[2] fa/fa=obese Zucker rat, lean=lean Zucker rat, faZD=fa/fa zinc deficient diet, faZC=fa/fa zinc control diet, faPW=fa/fa pair-weighed, faZS=fa/fa zinc supplemented diet, lnZD=lean zinc deficient diet, lnZC=lean zinc control diet, lnPW=lean pair-weighed and lnZS=lean zinc supplemented diet.

Table 6. Effects of dietary zinc on weekly body weight gain of obese (fa/fa) and lean Zucker rats[1]

	Dietary Treatment Groups[2]							
	fa/fa				lean			
	faZD	faZC	faPW	faZS	lnZD	lnZC	lnPW	lnZS
Week 1 Weight Gain (g)	64.6^{a} ±4.3	67.0^{a} ±4.9	64.6^{a} ±5.4	63.9^{a} ±3.4	51.4^{b} ±2.0	52.0^{b} ±0.8	55.7^{ab} ±3.6	59.7^{ab} ±3.1
Week 2 Weight Gain (g)	77.3^{a} ±4.6	69.9^{a} ±6.2	77.6^{a} ±2.9	75.4^{a} ±2.4	41.3^{b} ±1.6	48.6^{b} ±4.7	46.4^{b} ±1.1	49.3^{b} ±1.9
Week 3 Weight Gain (g)	46.0^{b} ±7.4	59.0^{a} ±4.3	53.9^{ab} ±2.3	56.3^{ab} ±2.4	22.3^{c} ±3.0	32.1^{c} ±4.7	30.2^{c} ±2.5	28.7^{c} ±2.8
Week 4 Weight Gain (g)	61.3^{a} ±2.2	63.3^{a} ±3.4	56.8^{a} ±6.1	61.2^{a} ±3.1	34.5^{b} ±3.7	32.4^{b} ±2.5	19.9^{b} ±6.6	36.0^{b} ±2.8
Week 5 Weight Gain (g)	51.5^{a} ±3.4	51.3^{a} ±1.6	32.3^{b} ±7.3	52.2^{a} ±3.0	24.9^{b} ±2.2	24.9^{b} ±3.3	30.6^{b} ±2.8	25.5^{b} ±2.7
Week 6 Weight Gain (g)	26.3^{a} ±2.0	32.5^{a} ±1.6	33.7^{a} ±1.7	28.5^{a} ±1.4	9.3^{b} ±4.1	4.4^{b} ±2.6	6.4^{b} ±5.0	10.0^{b} ±3.9
Week 7 Weight Gain (g)	38.7^{a} ±3.5	36.2^{ab} ±1.3	42.8^{a} ±3.0	37.5^{ab} ±3.0	21.8^{c} ±1.4	29.3^{bc} ±3.1	25.9^{c} ±2.8	23.8^{c} ±4.0
Week 8 Weight Gain (g)	18.6^{ab} ±5.5	20.6^{a} ±3.0	25.4^{a} ±4.3	26.1^{a} ±2.9	14.9^{abc} ±2.8	5.2^{bc} ±4.6	2.8^{c} ±7.6	14.3^{abc} ±3.7
Week 9 Weight Gain (g)	15.6^{ab} ±6.1	8.5^{ab} ±5.6	1.5^{b} ±6.7	5.8^{ab} ±2.2	7.8^{ab} ±4.7	17.0^{ab} ±5.9	20.6^{a} ±8.4	13.7^{ab} ±5.0

[1] Values represent the mean ± SEM for n=7 rats. Different superscript letters indicate significant differences between means within a given week as determined by Duncan's multiple range test.

[2] fa/fa=obese Zucker rat, lean=lean Zucker rat, faZD=fa/fa zinc deficient diet, faZC=fa/fa zinc control diet, faPW=fa/fa pair-weighed, faZS=fa/fa zinc supplemented diet, lnZD=lean zinc deficient diet, lnZC=lean zinc control diet, lnPW=lean pair-weighed and lnZS=lean zinc supplemented diet.

a significantly higher pancreas weight in the lnZC, lnPW and lnZS groups compared to the faZD and faZC groups. Calculation of pancreas weight in relation to body weight also demonstrated a significant genotype effect (Appendix B). The fa/fa rats had a 51% lower relative pancreas weights compared to the lean rats (0.22 ± 0.0 vs. 0.45 ± 0.02 grams, respectively) but there were no differences among the treatment groups (Table 8).

There was a significant main effect of genotype on the spleen weights in this experiment (Appendix B). The fa/fa rats had significantly heavier spleens compared to lean rats (0.68 ± 0.02 vs. 0.56 ± 0.01 grams, respectively). In terms of dietary treatment, Table 8 shows that the lnZC spleen weights were significantly lighter than the faZS group. Compared to the results for total spleen weight, analysis of relative spleen weight indicated that the fa/fa rats had significantly smaller *relative* spleen weights compared to the lean rats (0.11 ± 0.0 vs. 0.15 ± 0.0 grams, respectively). There were no differences in relative spleen weight due to dietary zinc intervention among the fa/fa or lean rats.

In summary, the kidney, small intestine and spleen weights were significantly heavier in the fa/fa rats. However, when organ weights (with the exception of the pancreas) are expressed in relation to body weight, the relative weights are significantly lower in the fa/fa rats. In regards to the pancreas weight, this organ was significantly lighter in the fa/fa rats whether expressed as total wet weight or relative to body weight.

Liver Weight and Liver Lipid

Liver weights and liver lipid measurements for all treatment groups are shown in Table 9. There was a significant main effect of genotype on liver weight (Appendix C). Fa/fa rats had significantly higher liver weights compared to lean rats (30.7 ± 1.2 vs. 11.7 ± 0.3 grams wet weight at termination, respectively), representing a 2.6 fold higher liver

weight in the fa/fa group. In terms of diet, the faPW group had significantly higher liver weights compared to the faZC group (34.3 ± 1.5 vs. 28.2 ± 3.1 grams, respectively). Relative liver weight, calculated as liver weight per 100 grams of body weight, is a useful measurement as it accounts for differences in body weight among the treatment groups. The fa/fa rats had greater relative liver weights compared to the lean rats (5.02 ± 0.18 vs. 3.18 ± 0.05 g/100 grams body weight, respectively), representing a 1.6 fold higher relative liver weight in the fa/fa group. When the different treatment groups were compared, the faPW rats had greater relative liver weights compared to the faZD, faZC and faZS groups, and there were no differences among the lean rats.

There was a significant main effect of genotype on liver lipid concentration in this experiment (Appendix C). The higher liver weights observed in the fa/fa rats were associated with significantly higher liver lipid concentrations (Table 9 and Figure 2). The fa/fa rats had 4.8 fold higher lipid per gram of liver than the lean rats (198.0 ± 9.8 vs. 40.9 ± 0.8 mg/g liver, respectively). Furthermore, a greater relative liver weight observed in the faPW was associated with significantly higher liver lipid concentration compared to the faZC group, but no differences were found among the lean rats.

Analysis of total liver lipid content revealed a 13.3 fold greater total lipid in the livers of fa/fa animals compared to lean animals (6.27 ± 0.46 vs. 0.47 ± 0.01 grams, respectively), although a significant effect of dietary treatment on total liver lipid content was only observed in the fa/fa group (Table 9 and Figure 3). The faPW rats had 7.93 ± 0.88 grams total lipid per liver compared to 5.15 ± 1.16 g in the faZC group and 5.84 ± 0.60 grams in the faZS group.

Liver lipid content was used to calculate the relative liver lipid content. This

Table 7. Effects of dietary zinc on kidney weight, and small intestine weight and length of obese (fa/fa) and lean Zucker rats after 9 weeks of dietary treatment[1]

	Dietary Treatment Groups[2]							
	fa/fa				lean			
	faZD	faZC	faPW	faZS	lnZD	lnZC	lnPW	lnZS
Kidney Weight (g)	3.96^{a} ±0.19	3.97^{a} ±0.23	4.27^{a} ±0.11	4.06^{a} ±0.15	2.91^{b} ±0.06	3.07^{b} ±0.06	2.87^{b} ±0.08	3.06^{b} ±0.08
Relative Kidney Weight (g/100 g body weight)	0.65^{bc} ±0.01	0.64^{c} ±0.02	0.72^{b} ±0.03	0.66^{bc} ±0.02	0.84^{a} ±0.03	0.84^{a} ±0.03	0.80^{a} ±0.03	0.79^{a} ±0.02
Small Intestine Weight (g)	8.04^{a} ±0.31	7.91^{a} ±0.40	8.36^{a} ±0.54	8.46^{a} ±0.26	5.59^{b} ±0.25	5.66^{b} ±0.02	5.80^{b} ±0.21	6.09^{b} ±0.20
Relative Small Intestine Weight (g/100 g body weight)	1.32^{c} ±0.03	1.27^{c} ±0.04	1.40^{bc} ±0.09	1.37^{c} ±0.04	1.59^{a} ±0.04	1.54^{ab} ±0.06	1.62^{a} ±0.05	1.57^{a} ±0.04
Small Intestine Length (cm)	91.6^{a} ±4.2	87.6^{a} ±4.8	85.7^{ab} ±6.6	92.0^{a} ±6.5	79.9^{ab} ±4.3	70.9^{b} ±4.3	77.3^{ab} ±3.0	77.0^{ab} ±2.9

[1] Values represent the mean ± SEM for n=7 rats. Different superscript letters indicate significant differences between means as determined by Duncan's multiple range test.

[2] fa/fa=obese Zucker rat, lean=lean Zucker rat, faZD=fa/fa zinc deficient diet, faZC=fa/fa zinc control diet, faPW=fa/fa pair-weighed, faZS=fa/fa zinc supplemented diet, lnZD=lean zinc deficient diet, lnZC=lean zinc control diet, lnPW=lean pair-weighed and lnZS=lean zinc supplemented diet.

Table 8. Effects of dietary zinc on pancreas and spleen weight of obese (fa/fa) and lean Zucker rats after 9 weeks of dietary treatment[1]

	Dietary Treatment Groups[2]							
	fa/fa				lean			
	faZD	faZC	faPW	faZS	lnZD	lnZC	lnPW	lnZS
Pancreas weight (g)	1.21^{b} ±0.09	1.26^{b} ±0.11	1.43^{ab} ±0.10	1.40^{ab} ±0.10	1.57^{ab} ±0.18	1.63^{a} ±0.14	1.70^{a} ±0.13	1.73^{a} ±0.04
Relative Pancreas Weight (g/100 g body weight)	0.20^{b} ±0.02	0.20^{b} ±0.02	0.24^{b} ±0.02	0.23^{b} ±0.01	0.45^{a} ±0.05	0.44^{a} ±0.03	0.48^{a} ±0.05	0.45^{a} ±0.01
Spleen Weight (g)	0.66^{ab} ±0.04	0.66^{ab} ±0.05	0.66^{ab} ±0.05	0.73^{a} ±0.03	0.59^{bc} ±0.01	0.53^{c} ±0.02	0.56^{bc} ±0.02	0.56^{bc} ±0.03
Relative Spleen Weight (g/100 g body weight)	0.11^{b} ±0.0	0.11^{b} ±0.01	0.11^{b} ±0.01	0.12^{b} ±0.01	0.17^{a} ±0.01	0.14^{a} ±0.01	0.16^{a} ±0.01	0.15^{a} ±0.01

[1] Values represent the mean ± SEM for n=7 rats. Different superscript letters indicate significant differences between means as determined by Duncan's multiple range test.

[2] fa/fa=obese Zucker rat, lean=lean Zucker rat, faZD=fa/fa zinc deficient diet, faZC=fa/fa zinc control diet, faPW=fa/fa pair-weighed, faZS=fa/fa zinc supplemented diet, lnZD=lean zinc deficient diet, lnZC=lean zinc control diet, lnPW=lean pair-weighed and lnZS=lean zinc supplemented diet.

measurement takes into account differences in liver weights among the treatment groups and thus provides a more accurate representation of differences in liver lipid profile. There was a significant genotype effect on relative liver lipid content (0.65 ± 0.02 vs. 0.36 ± 0.01 g/100 g liver in fa/fa and lean rats, respectively), with a 1.8 fold higher relative liver lipid content observed in the fa/fa rats. However, despite significant differences in liver lipid concentration and total liver lipid content observed within the fa/fa treatment groups (Figure 2 and Figure 3), there were no differences observed in relative liver lipid content (Table 9 and Figure 4).

Femur, Liver and Serum Zinc

Femur, liver and serum zinc are indices of zinc status. Table 10 summarizes the effects of dietary zinc on these parameters in fa/fa and lean Zucker rats after 9 weeks of dietary treatment. Measurements of femur length and femur dry weight were also recorded and analyzed. Femur weight were different by genotype (Appendix D). The fa/fa femurs were significantly lighter in weight compared to the lean rats (0.52 ± 0.01 vs. 0.55 ± 0.01 grams, respectively) and the lnZC femurs were heavier than the faPW femurs (Table 10). There was a significant main effect of genotype on the length of the femurs (Appendix D). The fa/fa rats had significantly shorter femurs compared to the lean rats (3.41 ± 0.02 vs. 3.64 ± 0.03 cm, respectively). In terms of dietary treatment groups, the faPW rats had significantly shorter femurs compared to the faZC and faZS groups and all lean rats.

Femur zinc concentration is an indicator of long-term zinc status. After 9 weeks of dietary intervention, there was a significant genotype and dietary zinc effect on femur zinc concentration (Appendix D). The fa/fa rats had approximately 18% higher zinc

concentrations in femur compared to lean rats (340 ± 11 vs. 288 ± 13 *u*g/g dry femur weight, respectively). A comparison of all 8 treatment groups demonstrated differences in femur zinc concentration in response to dietary zinc intervention. Among the fa/fa rats, the faZD group had significantly lower (32-34%) femur zinc concentration than the faZC, faPW and faZS groups (Figure 5). Similarly, the lnZD group had a significantly lower (43%) femur zinc concentration compared to the lnZC, lnPW and lnZS rats. In both the fa/fa and lean rats, the ZS groups were not significantly higher than the ZC groups, possibly indicating that the level of zinc used in the ZS group (150 ppm) was not adequate to elevate the femur zinc stores of the rats. Nevertheless, the marginal zinc deficiency (5 ppm) used in the experiment was an adequate restriction to affect femur zinc stores of both the fa/fa and lean rats. Interestingly, the femur zinc concentration in the faZD rats was significantly higher (26.1%) than the lnZD rats.

Liver zinc is another indicator of long term body pools of zinc. There was a significant main effect of genotype on liver zinc concentration in this experiment (Appendix D). In contrast to the results for femur zinc concentration, the fa/fa rats had lower liver zinc concentration compared to the lean rats (47.6 ± 2.1 vs. 94.1 ± 2.2 *u*g/g dry liver weight, respectively). The lean rats had a 49.4% higher liver zinc concentration than the fa/fa rats, indicating that the pools of zinc in the fa/fa rats (femur and liver) are different in terms of zinc retention. Whereas the fa/fa rats retained more zinc (18%) in the femurs compared to lean rats, hepatic zinc retention was significantly lower in the fa/fa rats compared to lean rats.

Table 9. Effects of dietary zinc on liver weight and liver lipid of obese (fa/fa) and lean Zucker rats after 9 weeks of dietary treatment[1]

	Dietary Treatment Groups[2]							
	fa/fa				lean			
	faZD	faZC	faPW	faZS	lnZD	lnZC	lnPW	lnZS
Liver Weight (g)	29.6^{ab} ±2.6	28.2^{b} ±3.1	34.3^{a} ±1.5	30.7^{ab} ±1.3	11.5^{c} ±0.5	11.8^{c} ±0.5	10.9^{c} ±0.4	12.4^{c} ±0.7
Relative Liver Weight (g/100 g body weight)	4.84^{b} ±0.33	4.51^{b} ±0.42	5.75^{a} ±0.31	4.99^{b} ±0.26	3.30^{c} ±0.10	3.19^{c} ±0.06	3.03^{c} ±0.07	3.21^{c} ±0.12
Liver Lipid Concentration (mg/g liver)	203.8^{ab} ±13.1	171.1^{b} ±26.1	228.0^{a} ±18.1	189.2^{ab} ±16.5	41.3^{c} ±1.3	39.0^{c} ±1.8	41.0^{c} ± 2.1	42.4^{c} ±1.5
Liver Lipid Content (g)	6.17^{ab} ±0.85	5.15^{b} ±1.16	7.93^{a} ±0.88	5.84^{b} ±0.60	0.47^{c} ±0.02	0.46^{c} ±0.02	0.45^{c} ±0.02	0.53^{c} ±0.03
Relative Liver Lipid Content (g/100 g liver)	0.70^{a} ±0.04	0.61^{a} ±0.06	0.66^{a} ±0.04	0.62^{a} ±0.06	0.36^{b} ±0.02	0.34^{b} ±0.03	0.38^{b} ±0.02	0.35^{b} ±0.02

[1] Values represent the mean ± SEM for n=7 rats. Different superscript letters indicate significant differences between means as determined by Duncan's multiple range test.

[2] fa/fa=obese Zucker rat, lean=lean Zucker rat, faZD=fa/fa zinc deficient diet, faZC=fa/fa zinc control diet, faPW=fa/fa pair-weighed, faZS=fa/fa zinc supplemented diet, lnZD=lean zinc deficient diet, lnZC=lean zinc control diet, lnPW=lean pair-weighed and lnZS=lean zinc supplemented diet.

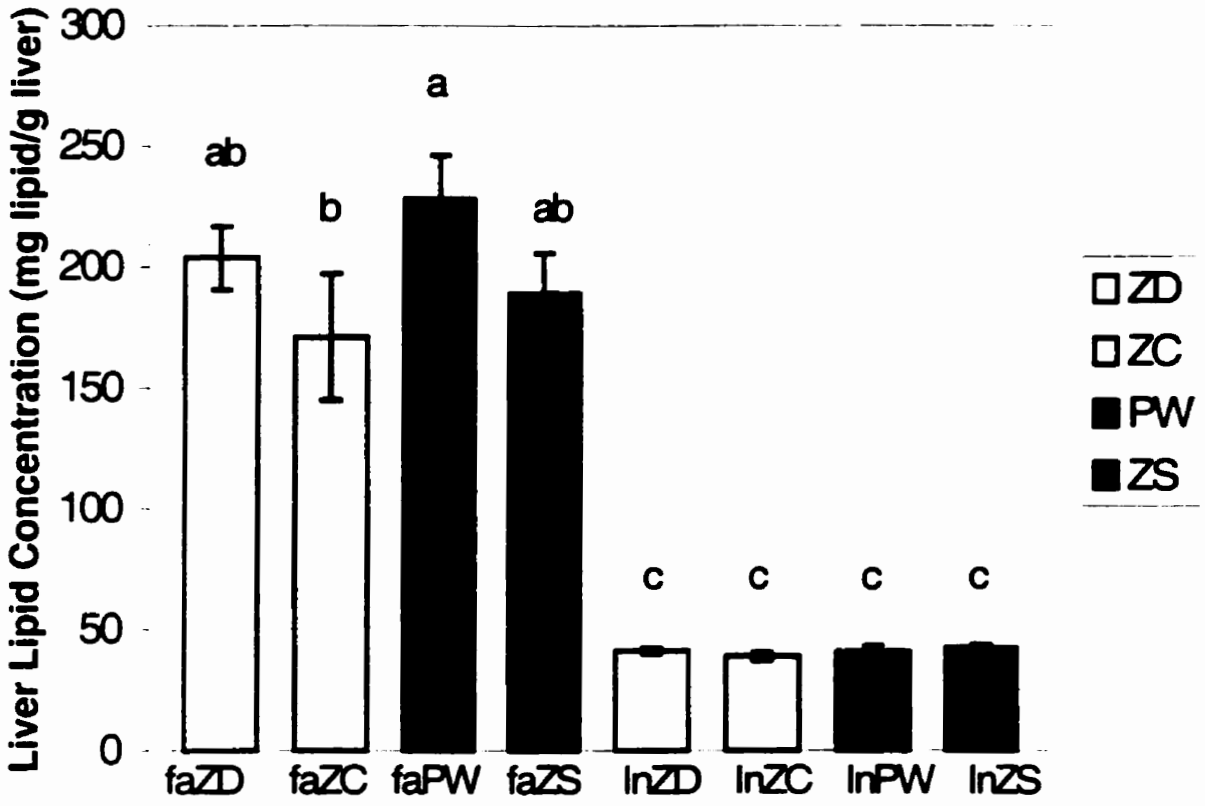


Figure 2. The effects of dietary zinc on liver lipid concentration of obese (fa/fa) and lean Zucker rats after 9 weeks of dietary treatment. Treatment groups were faZD=fa/fa zinc deficient diet, faZC=fa/fa zinc control diet, faPW=fa/fa pair-weighed, faZS=fa/fa zinc supplemented diet, lnZD=lean zinc deficient diet, lnZC=lean zinc control diet, lnPW=lean pair-weighed and lnZS=lean zinc supplemented diet. Each bar represents the mean liver lipid concentration ± SEM for n = 7 rats. Different lowercase letters indicate significant differences between means as determined by Duncan's multiple range test.

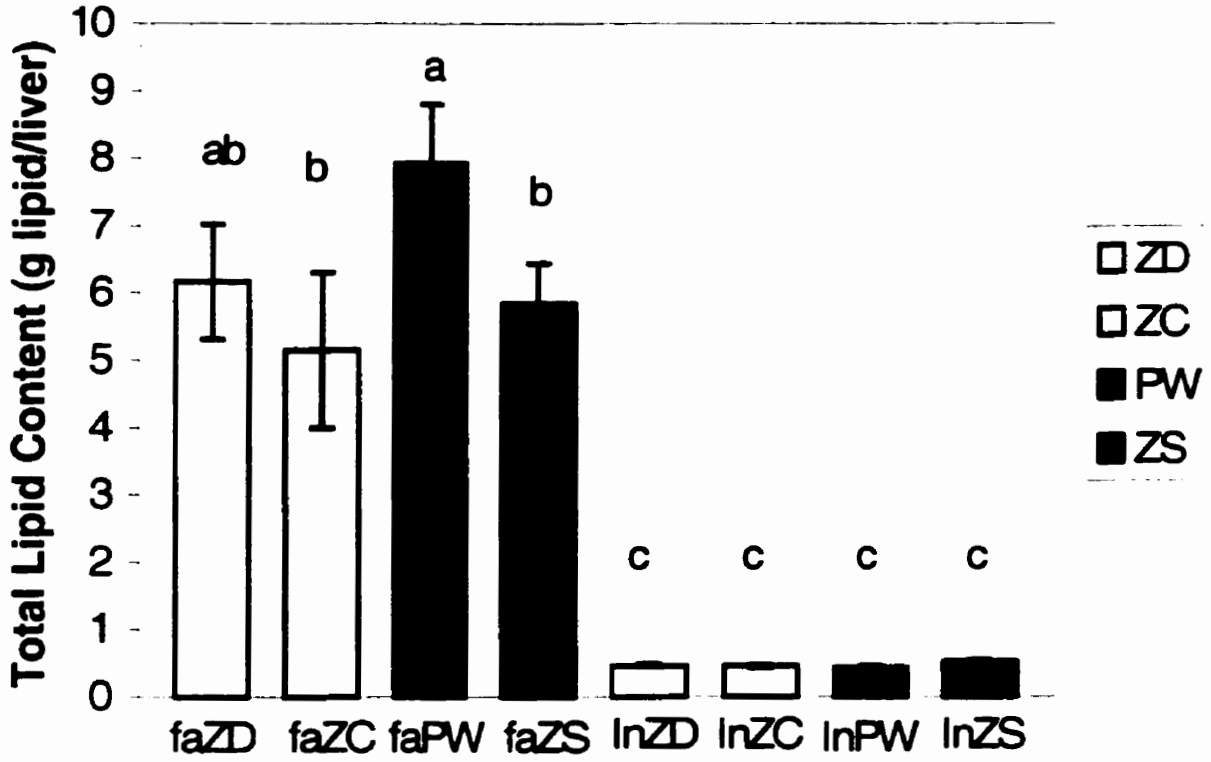


Figure 3. The effects of dietary zinc on total liver lipid content of obese (fa/fa) and lean Zucker rats after 9 weeks of dietary treatment. Treatment groups were faZD=fa/fa zinc deficient diet, faZC=fa/fa zinc control diet, faPW=fa/fa pair-weighed, faZS=fa/fa zinc supplemented diet, lnZD=lean zinc deficient diet, lnZC=lean zinc control diet, lnPW=lean pair-weighed and lnZS=lean zinc supplemented diet. Each bar represents the mean total liver lipid content ± SEM for n = 7 rats. Different lowercase letters indicate significant differences between means as determined by Duncan's multiple range test.

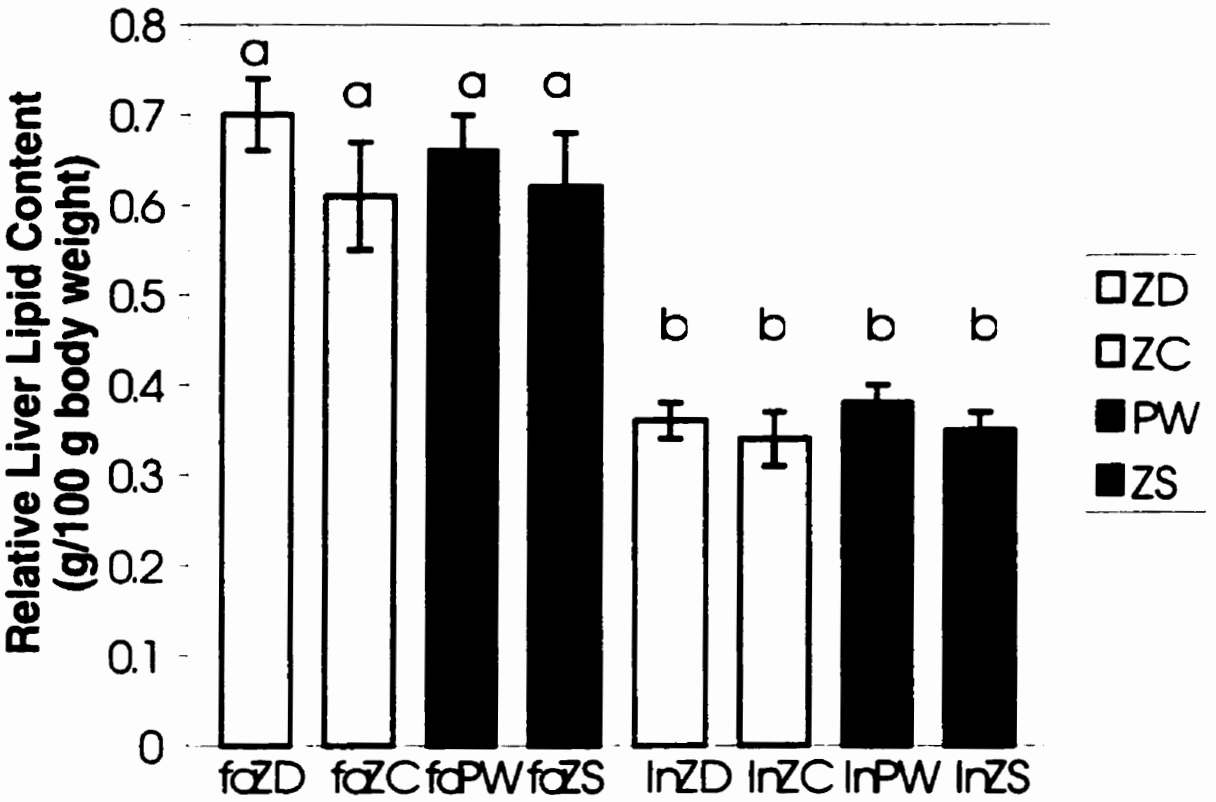


Figure 4. The effects of dietary zinc on relative liver lipid content of obese (fa/fa) and lean Zucker rats after 9 weeks of dietary treatment. Treatment groups were faZD=fa/fa zinc deficient diet, faZC=fa/fa zinc control diet, faPW=fa/fa pair-weighed, faZS=fa/fa zinc supplemented diet, lnZD=lean zinc deficient diet, lnZC=lean zinc control diet, lnPW=lean pair-weighed and lnZS=lean zinc supplemented diet. Each bar represents the mean relative liver lipid content ± SEM for n=7 rats. Different lowercase letters indicate significant differences between means as determined by Duncan's multiple range test.

There were no significant differences in liver zinc concentration among the dietary treatment groups in both the fa/fa and lean rats (Figure 6). Therefore, dietary zinc intervention affected femur zinc status but not liver zinc concentrations.

As indicated in Table 9, the fa/fa rats had significantly heavier total liver weights and relative liver weights. Therefore, to take into account differences in liver weights, the total liver zinc content per liver and the relative liver zinc content was calculated and analyzed. There was a significant main effect of genotype on the total liver zinc content (Appendix D). The fa/fa rats had significantly higher total liver zinc content compared to the lean rats (585 ± 15 vs. 301 ± 9 *u*g/liver, respectively). When the different treatment groups are compared, the faZC group had significantly less total liver zinc content compared to the faPW and faZS groups and no differences were found among the lean rats (Table 10). Genotype also had a significant main effect on the relative liver zinc content (Appendix D). The relative liver zinc content in the fa/fa rats was lower (2.00 ± 0.0 *u*g/100 mg liver) compared to the lean rats (3.00 ± 0.0 *u*g/100 mg liver). Therefore, even when differences in liver weights are considered, the hepatic zinc storage in the fa/fa rats remained significantly lower then lean rats. In terms of dietary zinc, there were no differences in relative liver zinc content among either the fa/fa or lean rats.

Another indicator of zinc status is serum zinc concentration, which reflects short-term zinc status. There was a significant main effect of genotype and dietary zinc on serum zinc concentrations in this experiment (Appendix D). The fa/fa rats had significantly higher (23.2%) serum zinc concentrations compared to the lean rats (2.41 ± 0.07 vs. 1.85 ± 0.04 *u*g/ml, respectively). In terms of diet, the lnZD rats had a significantly lower serum zinc concentration than the lnZS group (Figure 7). However,

serum zinc concentration in the faZD group was significantly *greater* than the faZC group and surprisingly similar to the faZS group. Furthermore, the serum zinc concentration in the faZD rats was higher (31.8%) compared to the lnZD rats. Therefore, with the exception of liver zinc concentration, the fa/fa rats had higher zinc concentration in storage pools and in circulation compared to lean rats, and a zinc deficient diet appears to have different effects on the short term status of the fa/fa rat compared to lean rats. To explore the potential association between zinc storage pools in the body and circulating zinc in the serum, the data were analyzed by Pearson's Product correlation. There was a nonsignificant correlation between serum and femur zinc for the lean and fa/fa rats. Furthermore, a non significant correlation was found between serum zinc and liver zinc concentrations for both genotype groups (Figure 8). Therefore, the lower liver zinc concentration observed among the fa/fa rats was not related to higher serum zinc concentrations.

Femur Calcium and Phosphorus

Femur calcium and femur phosphorus concentrations were measured to assess the mineral status of the fa/fa and lean rats. There was a significant effect of genotype, zinc and the interaction of genotype and zinc for femur calcium (Appendix D). The fa/fa rats had significantly higher femur calcium concentrations compared to lean rats (456 ± 27 vs. 353 ± 7 mg/g dry weight, respectively) representing a 22.6% higher femur calcium concentration in the fa/fa rats. In terms of the main effect of zinc, there was a significantly higher femur calcium concentration in the ZD group compared to the ZC and ZS dietary treatment groups (520 ± 48 vs. 361 ± 7 and 381 ± 13 mg/g dry weight, respectively). Table 11 illustrates the interaction of genotype and zinc on femur calcium

concentrations. The faZD rats had significantly higher femur calcium concentration compared to all other fa/fa and lean rats. The femur calcium concentration was similar among the faZC, faPW and faZS groups and among the lean rats. Therefore, it appears that a marginally zinc deficient diet positively influences (elevates) the femur calcium stores of the fa/fa (obese) Zucker rat model, but not their lean counterparts.

Genotype, dietary zinc and the interaction of genotype and zinc had significant main effects on femur phosphorous concentrations (Appendix D). The femur phosphorous concentration was significantly higher in the fa/fa rats compared to the lean rats (217 ± 12 vs. 172 ± 4 mg/g dry weight, respectively) representing 16.5% higher femur phosphorous concentration in the fa/fa rat model. Similar to the results for femur calcium concentrations, there was a significantly higher femur phosphate concentration with the ZD diet compared to the ZC and ZS intervention (245 ± 22 vs. 174 ± 4 and 186 ± 5 mg/g dry femur weight, respectively). The significant interaction of genotype and zinc is documented in Table 11. The faZD rats showed the highest femur phosphate concentration compared to all other rats (fa/fa and lean) in the experiment. There were no significant differences among the faZC, faPW and faZS rats, or among the lean dietary treatment groups.

In summary, the fa/fa rats fed a marginally zinc deficient diet (5 ppm) showed a significantly higher femur calcium and phosphorus concentrations. However, this effect of the ZD diet was not observed in the lean rats.

Liver Copper

Liver copper concentration was analyzed to assess the toxicity of the zinc supplemented diet used in this experiment. The results for liver copper concentration,

total copper per liver and the relative liver copper are shown in Table 11. There was a significant main effect of genotype on liver copper concentrations (Appendix C). The fa/fa rats had significantly lower (28%) liver copper concentrations compared to their lean counterparts (39.0 ± 2.4 vs. 54.2 ± 2.2 *u*g/g dry weight, respectively) and no differences were observed among dietary treatment groups (Table 11). There was a significant main effect of genotype on total copper content per liver (Appendix C). On the contrary to the results for liver copper concentrations, the fa/fa rats had significantly more (61%) total copper per liver compared to the lean rats (492 ± 33 vs. 192 ± 20 mg/liver, respectively) and no differences were observed among dietary treatment groups, as illustrated in Table 11. Despite a significant genotype difference in liver copper concentrations and total liver copper content, there were no differences in liver copper status between the fa/fa and lean rats when expressed as mg copper per 100 mg liver. The fa/fa rats had 1.62 ± 0.10 mg copper/100 mg liver which was similar to 1.64 ± 0.16 mg copper/100 mg liver observed in the lean rats. Similarly, there were no differences in relative liver copper content due to dietary zinc.

Table 10. Effects of dietary zinc on femur weight and length, and femur, liver and serum zinc of obese (fa/fa) and lean Zucker rats after 9 weeks of dietary treatment[1]

	Dietary Treatment Groups[2]							
	fa/fa				lean			
	faZD	faZC	faPW	faZS	lnZD	lnZC	lnPW	lnZS
Femur Weight (g, dry weight)	0.52^{ab} ±0.01	0.53^{ab} ±0.02	0.50^{b} ±0.02	0.52^{ab} ±0.02	0.53^{ab} ±0.01	0.57^{a} ±0.02	0.55^{ab} ±0.02	0.55^{ab} ±0.03
Femur Length (cm)	3.41^{de} ±0.03	3.49^{bcd} ±0.03	3.27^{e} ±0.06	3.47^{cd} ±0.03	3.63^{abc} ±0.09	3.70^{a} ±0.04	3.59^{abc} ±0.03	3.64^{ab} ±0.06
Femur Zinc Concentration (*u*g/g dry weight)	249^{d} ±7	358^{ab} ±8	375^{a} ±12	379^{a} ±13	184^{e} ±8	322^{c} ±4	324^{c} ±8	336^{bc} ±15
Liver Zinc Concentration (*u*g/g dry weight)	48.7^{b} ±5.1	50.2^{b} ±6.1	42.2^{b} ±2.3	49.1^{b} ±2.6	94.9^{a} ±5.0	98.3^{a} ±6.1	91.4^{a} ±3.6	91.7^{a} ±2.1
Total Liver Zinc Content (*u*g/liver)	571^{ab} ±32	537^{b} ±32	622^{a} ±23	610^{a} ±21	291^{c} ±20	319^{c} ±23	280^{c} ±10	314^{c} ±14
Relative Liver Zinc Content (*u*g/100 mg liver)	2.0^{b} ±0.0	2.0^{b} ±0.0	2.0^{b} ±0.0	2.0^{b} ±0.0	3.0^{a} ±0.0	3.0^{a} ±0.0	3.0^{a} ±0.0	3.0^{a} ±0.0
Serum Zinc Concentration (*u*g/ml)	2.45^{a} ±0.09	2.04^{b} ±0.06	2.52^{a} ±0.14	2.61^{a} ±0.19	1.67^{c} ±0.05	1.89^{bc} ±0.08	1.83^{bc} ±0.04	2.00^{b} ±0.05

[1] Values represent the mean ± SEM for n=7 rats except n=5 for lnZS femur measurements. Different superscript letters indicate significant differences between means as determined by Duncan's multiple range test.

[2] fa/fa=obese Zucker rat, lean=lean Zucker rat, faZD=fa/fa zinc deficient diet, faZC=fa/fa zinc control diet, faPW=fa/fa pair-weighed, faZS=fa/fa zinc supplemented diet, lnZD=lean zinc deficient diet, lnZC=lean zinc control diet, lnPW=lean pair-weighed and lnZS=lean zinc supplemented diet.

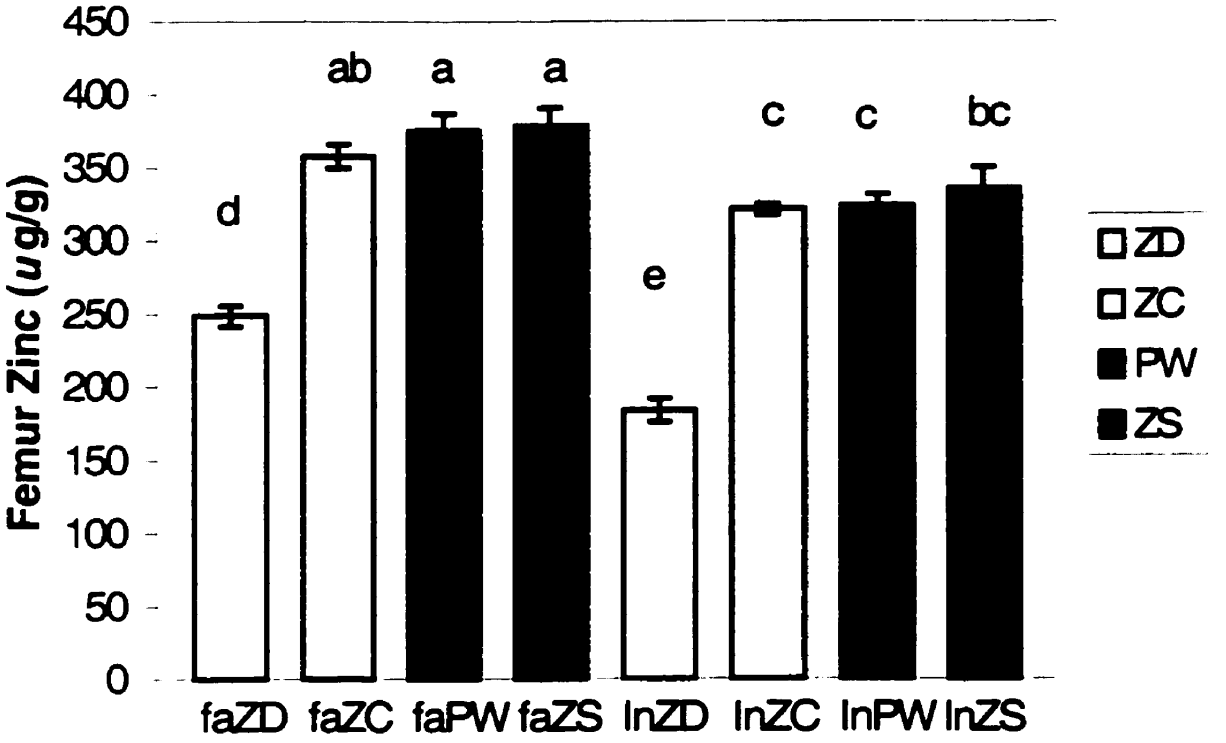


Figure 5. The effect of dietary zinc on femur zinc concentration of obese (fa/fa) and lean Zucker rats after 9 weeks of dietary treatment. Treatment groups were faZD=fa/fa zinc deficient diet, faZC=fa/fa zinc control diet, faPW=fa/fa pair-weighed, faZS=fa/fa zinc supplemented diet, lnZD=lean zinc deficient diet, lnZC=lean zinc control diet, lnPW=lean pair-weighed and lnZS=lean zinc supplemented diet. Each bar represents the mean femur zinc concentration ± SEM for n=7 rats except n=5 for lnZS. Different lower case letters indicate significant differences between means as determined by Duncan's multiple range test.

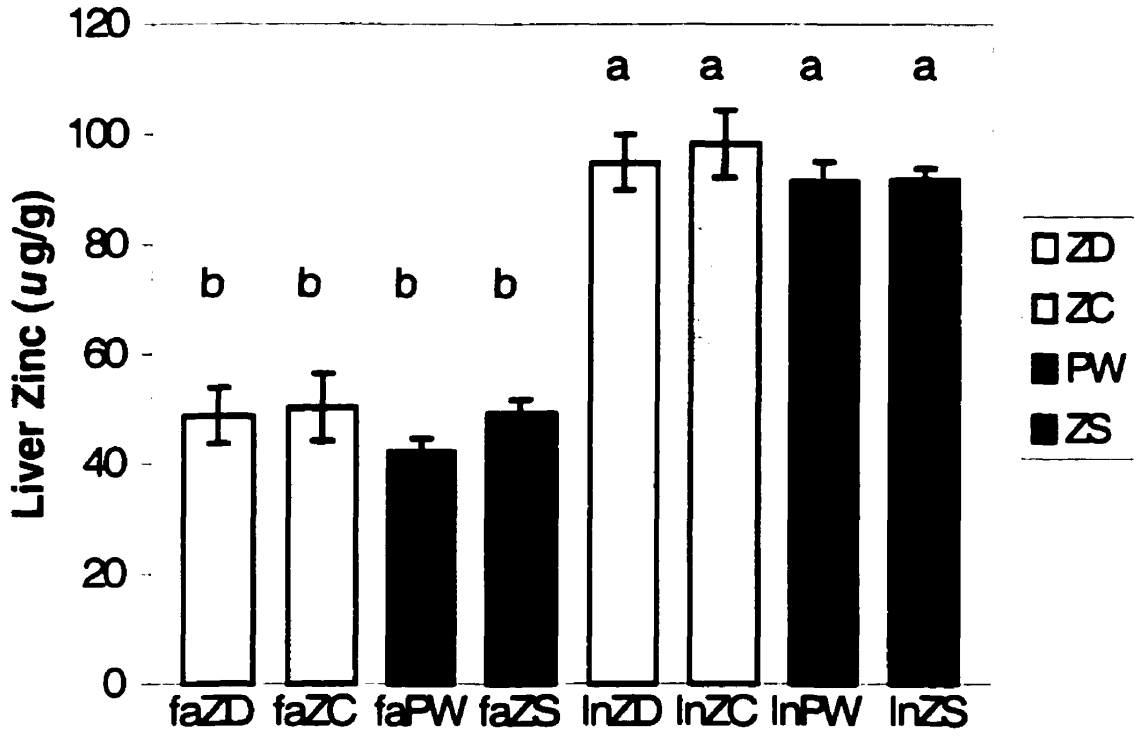


Figure 6. The effect of dietary zinc on liver zinc concentration of obese (fa/fa) and lean Zucker rats after 9 weeks of dietary treatment. Treatment groups were faZD=fa/fa zinc deficient diet, faZC=zinc control diet, faPW=fa/fa pair-weighed, faZS=fa/fa zinc supplemented diet, lnZD=lean zinc deficient diet, lnZC=lean zinc control diet, lnPW=lean pair-weighed and lnZS=lean zinc supplemented diet. Each bar represents the mean liver zinc concentration ± SEM for n=7 rats. Different lower case letters indicate significant differences between means as determined by Duncan's multiple range test.

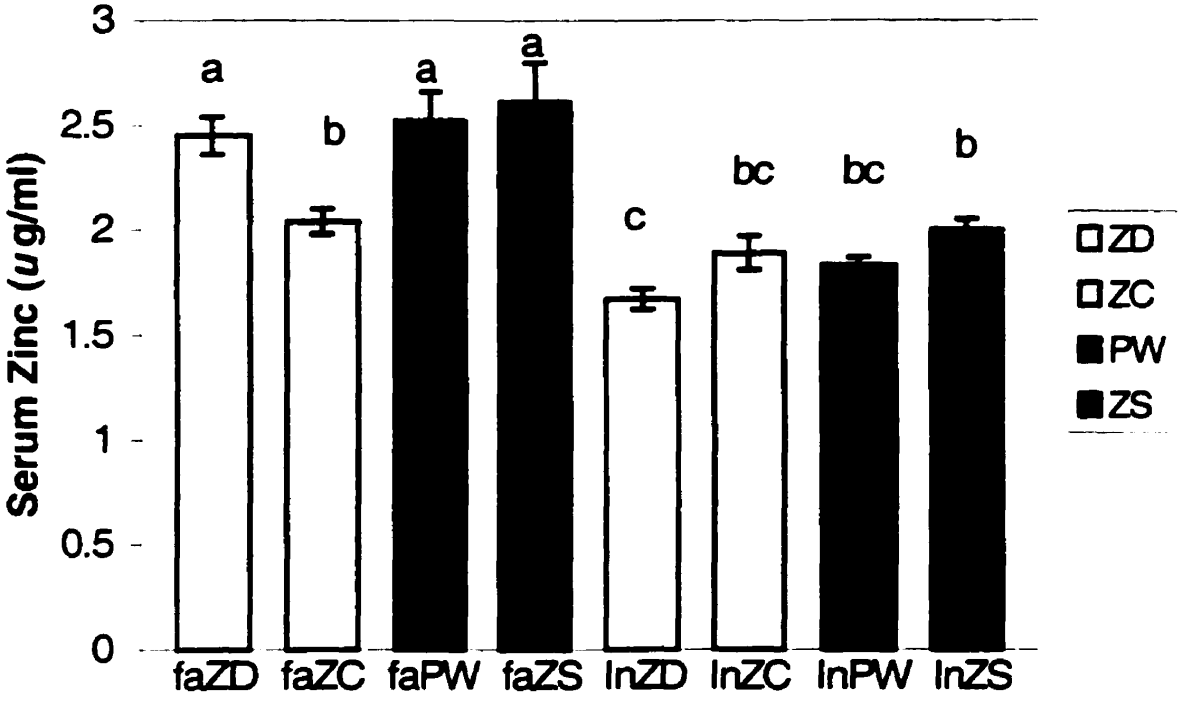
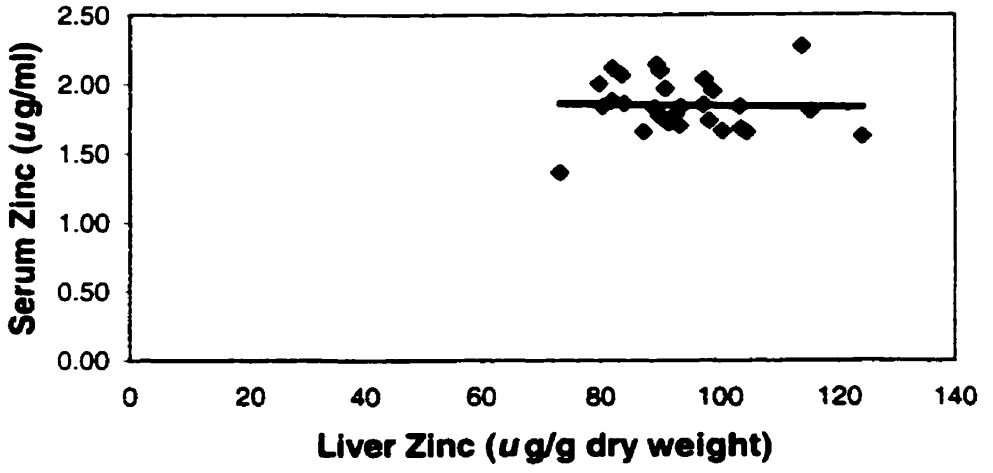


Figure 7. The effect of dietary zinc on serum zinc concentration of obese (fa/fa) and lean Zucker rats after 9 weeks of dietary treatment. Treatment groups were faZD=fa/fa zinc deficient diet, faZC=zinc control diet, faPW=fa/fa pair-weighed, faZS=fa/fa zinc supplemented diet, lnZD=lean zinc deficient diet, lnZC=lean zinc control diet, lnPW=lean pair-weighed and lnZS=lean zinc supplemented diet. Each bar represents the mean serum zinc concentration ± SEM for n=7 rats. Different lower case letters indicate significant differences between means as determined by Duncan's multiple range test.

a)



b)

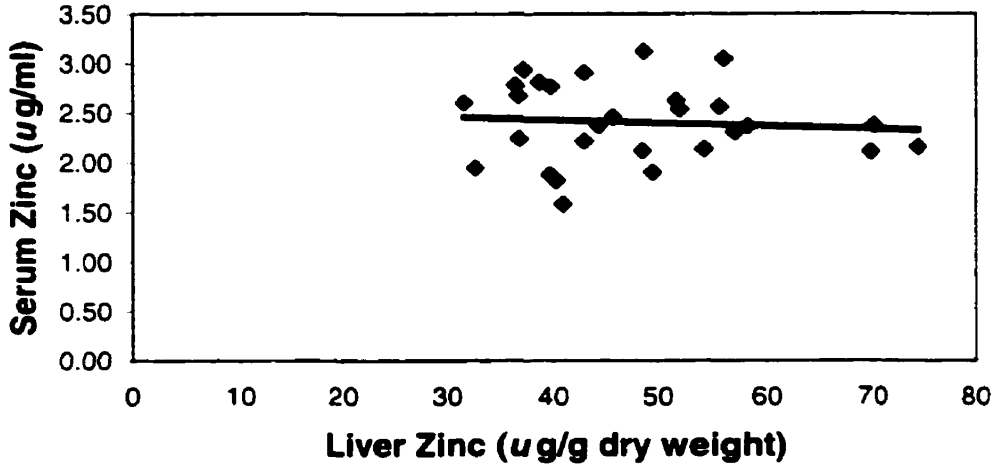


Figure 8. Scatter plot of serum zinc concentration vs. liver zinc concentration for a) lean rats and b) fa/fa rats. Data points are values obtained from individual n=28 rats for each genotype. Analysis by Pearson's correlation coefficient revealed a non significant correlation ($r = -0.04$, $p=0.84$ and $r=-0.08$, $p=0.66$ for lean and fa/fa rats, respectively).

Serum Insulin and Serum Glucose

The results for both serum insulin and serum glucose concentration in obese (fa/fa) and lean Zucker rats are illustrated in Table 12. There was a significant main effect of genotype on serum insulin (Appendix E). The fa/fa rats exhibited a 22 fold higher serum insulin concentration compared to the lean rats (41.9 ± 4.7 vs. 1.9 ± 0.1 ng/ml, respectively). In terms of diet, the faPW group had a significantly higher (46.3%) serum insulin concentration compared to the faZC group (54.6 ± 13.7 vs. 29.3 ± 5.9 ng/ml, respectively) despite both of these groups consuming the same level of dietary zinc (Figure 9). A similar effect of dietary treatment was not observed in the lean rats. The fa/fa rat is a hyperinsulinemic but normoglycemic animal model, and thus we did not expect to observe hyperglycemia in this experiment. However, we were still interested in measuring serum glucose and assessing any changes attributed to genotype or diet intervention. There was no main effect of genotype or diet on serum glucose concentration (Appendix E). Figure 10 illustrates the similarities in mean serum glucose concentration among the fa/fa and lean rats. The only significant difference among treatment groups was the significantly lower serum glucose concentration observed in the faZD rats compared to the lnZS rats (10.1 ± 0.8 vs. 13.2 ± 1.7 mmol/L, respectively). Therefore, the fa/fa rats in this experiment had a significantly higher serum insulin concentration compared to the lean rats although this did not translate into significant changes in serum glucose. Of particular interest is the observation of higher serum insulin concentrations in the faPW group that were not associated with a reduction in serum glucose.

Urinary Parameters: Volume, Creatinine, Zinc and Glucose

Urine was collected at week 0, 3, 6 and 9 of the experiment. The results for urine volume and urinary creatinine, zinc and glucose *at the end* of the 9 week experiment are shown in Table 13. There was no significant main effect of genotype or diet for urinary volume (Appendix E) collected at 9 weeks and no differences were observed among the dietary treatment groups in both the fa/fa and lean rats (Table 13). However, there was a significant main effect of genotype for urinary creatinine excretion in addition to a significant interaction of genotype and zinc at 9 weeks (Appendix E). The fa/fa rats excreted 31% less creatinine per 12 hours compared to the lean rats (2.96 ± 0.18 vs. 4.31 ± 0.29 mg/12 hours, respectively) but there were no differences due to dietary zinc (Table 13). Among the lean rats, the lnZC and lnPW groups excreted significantly more creatinine compared to the lnZD and lnZS groups. Therefore, it appears that a zinc control diet (30 ppm) interacted with the lean genotype Zucker rats to influence higher urine creatinine at week 9.

Genotype had a significant effect on urinary zinc excretion after 9 weeks of dietary zinc intervention (Appendix E). Compared to the results for urinary creatinine, the fa/fa rats excreted 84.6% more zinc compared to the lean rats (4.55 ± 0.77 vs. 0.7 ± 0.1 mg zinc/mg creatinine, respectively). In terms of dietary treatment, the faPW group excreted more zinc compared to the faZD, faZC and faZS groups and no differences were observed among the lean rats (Table 13 and Figure 11).

Table 11. Effects of dietary zinc on femur calcium and phosphorus, and liver copper of obese (fa/fa) and lean Zucker rats after 9 weeks of dietary treatment[1]

	Dietary Treatment Groups[2]							
	fa/fa				lean			
	faZD	faZC	faPW	faZS	lnZD	lnZC	lnPW	lnZS
Femur Calcium (mg/g, dry weight)	688^{a} ±19	360^{bc} ±16	374^{bc} ±13	404^{b} ±12	352^{c} ±15	358^{bc} ±14	352^{c} ±16	349^{c} ±19
Femur Phosphorus (mg/g dry weight)	321^{a} ±8	172^{bc} ±7	180^{bc} ±7	194^{b} ±5	168^{c} ±7	174^{bc} ±8	170^{c} ±8	176^{bc} ±9
Liver Copper Concentration (μg/g dry weight)	45.8^{abcd} ±6.7	38.2^{cd} ±5.5	33.2^{d} ±2.1	38.8^{bcd} ±2.53	57.0^{a} ±2.2	52.9^{ab} ±6.3	49.0^{abc} ±3.6	58.2^{a} ±4.4
Total Liver Copper (mg/liver)	574^{a} ±110	411^{a} ±57	489^{a} ±24	492^{a} ±42	250^{b} ±77	171^{b} ±20	149^{b} ±10	197^{b} ±12
Relative Liver Copper Content (mg/100 mg liver)	1.90^{a} ±0.30	1.54^{a} ±0.23	1.44^{a} ±0.09	1.60^{a} ±0.12	2.12^{a} ±0.59	1.46^{a} ±0.18	1.38^{a} ±0.09	1.62^{a} ±0.14

[1] Values represent the mean ± SEM for n=7 rats except n=5 for lnZS femur measurements and n=6 for lnZD liver copper measurements. Different superscript letters indicate significant differences between means as determined by Duncan's multiple range test.

[2] fa/fa=obese Zucker rat, lean=lean Zucker rat, faZD=fa/fa zinc deficient diet, faZC=fa/fa zinc control diet, faPW=fa/fa pair-weighed, faZS=fa/fa zinc supplemented diet, lnZD=lean zinc deficient diet, lnZC=lean zinc control diet, lnPW=lean pair-weighed and lnZS=lean zinc supplemented diet.

Urine glucose at the end of the experiment was also significantly influenced by genotype (Appendix E). There was not, however, any significant main effect of zinc per se or the interaction of genotype and zinc on urine glucose. The fa/fa rats excreted 99.7% more glucose compared to the lean rats (34.1 ± 11.6 vs. 0.10 ± 0.03 mg glucose/mg creatinine, respectively). In terms of dietary intervention, the faPW rats excreted more glucose than all other treatment groups (Table 13).

To understand how urine volume, creatinine, zinc and glucose changed during the 9 week experiment, urine was collected and analyzed at 3 time points. The results for urine volume at weeks 0, 3, 6 and 9 are shown in Table 14. In the fa/fa dietary treatment groups, the urine volume did not change throughout the experiment for the faZD, faZC and faZS rats. On the contrary, the faPW showed increased urine volume at week 9 compared to baseline. Among the lean rats, there were no differences in urine volume between time points in the lnZD and lnZS rats. However, the lnZC rats showed increased urine volume at week 3 compared to baseline and the lnPW rats showed increased in urine volume from baseline to week 6 and week 9 (Table 14).

The results for urinary creatinine at weeks 0, 3 and 6, in addition to week 9 are presented in Table 15. At weeks 6 and 9, urine creatinine was significantly higher compared to weeks 3 and baseline in the faZD and the faZS groups. This trend was different from that of the faZC rats where there were no differences in urine creatinine between time points. In the faPW group, there was significantly more creatinine excreted between weeks 0 and 6, but no differences from 3 weeks to the end of the experiment (9 weeks). The differences in the pattern of urine creatinine between the faZC and faPW rats were

Table 12. Effects of dietary zinc on serum insulin and serum glucose concentrations of obese (fa/fa) and lean Zucker rats after 9 weeks of dietary treatment[1]

	Dietary Treatment Groups[2]							
	fa/fa				lean			
	faZD	faZC	faPW	faZS	lnZD	lnZC	lnPW	lnZS
Serum Insulin (ng/ml)	47.4^{ab} ±6.1	29.3^{b} ±5.9	54.6^{a} ±13.7	36.2^{ab} ±8.8	1.7^{c} ±0.3	1.7^{c} ±0.3	1.9^{c} ±0.2	2.3^{c} ±0.3
Serum Glucose (mmol/L)	10.1^{b} ±0.8	11.9^{ab} ±0.5	11.2^{ab} ±1.1	11.5^{ab} ±0.4	11.4^{ab} ±0.7	11.4^{ab} ±0.6	11.5^{ab} ±0.3	13.2^{a} ±1.7

[1] Values represent the mean ± SEM for n=7 rats. Different superscript letters indicate significant differences between means as determined by Duncan's multiple range test.

[2] fa/fa=obese Zucker rat, lean=lean Zucker rat, faZD=fa/fa zinc deficient diet, faZC=fa/fa zinc control diet, faPW=fa/fa pair-weighed, faZS=fa/fa zinc supplemented diet, lnZD=lean zinc deficient diet, lnZC=lean zinc control diet, lnPW=lean pair-weighed and lnZS=lean zinc supplemented diet.

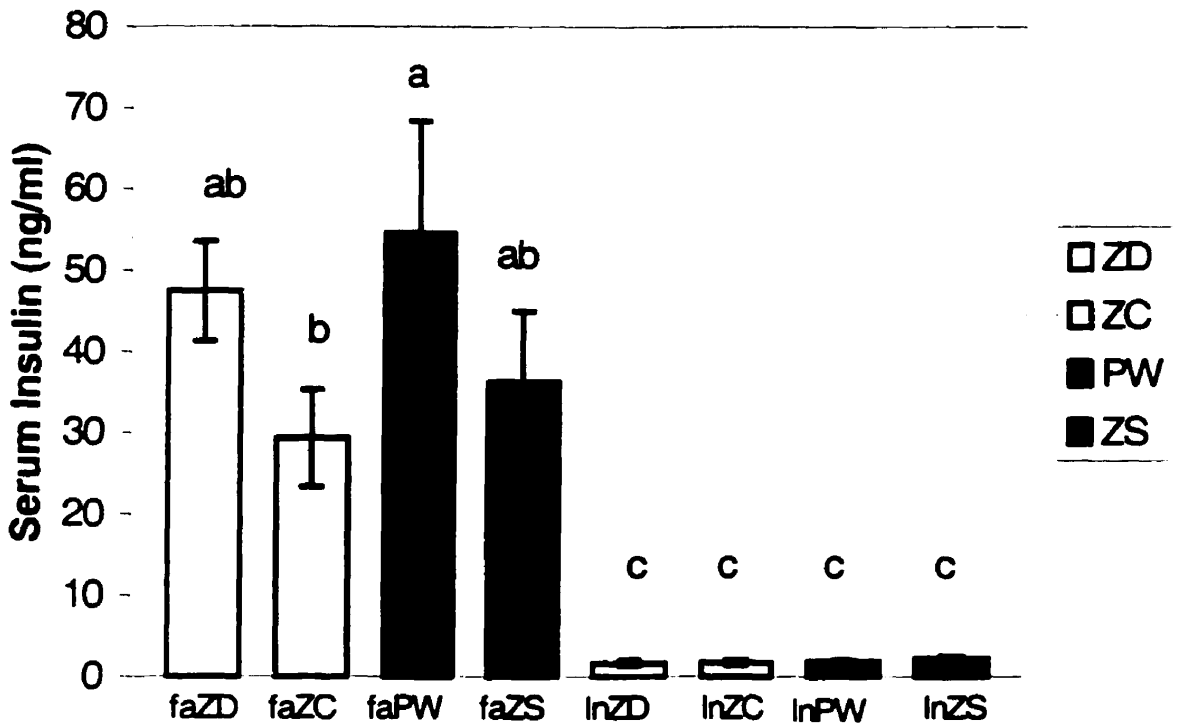


Figure 9. The effects of dietary zinc on serum insulin concentration of obese (fa/fa) and lean Zucker rats after 9 weeks of dietary treatment. Treatment groups were faZD=fa/fa zinc deficient diet, faZC=fa/fa zinc control diet, faPW=fa/fa pair-weighed, faZS=fa/fa zinc supplemented diet, lnZD=lean zinc deficient diet, lnZC=lean zinc control diet, lnPW=lean pair-weighed and lnZS=lean zinc supplemented diet. Each bar represents the mean serum insulin concentration ± SEM for n=7 rats. Different lowercase letters indicate significant differences between means as determined by Duncan's multiple range test.

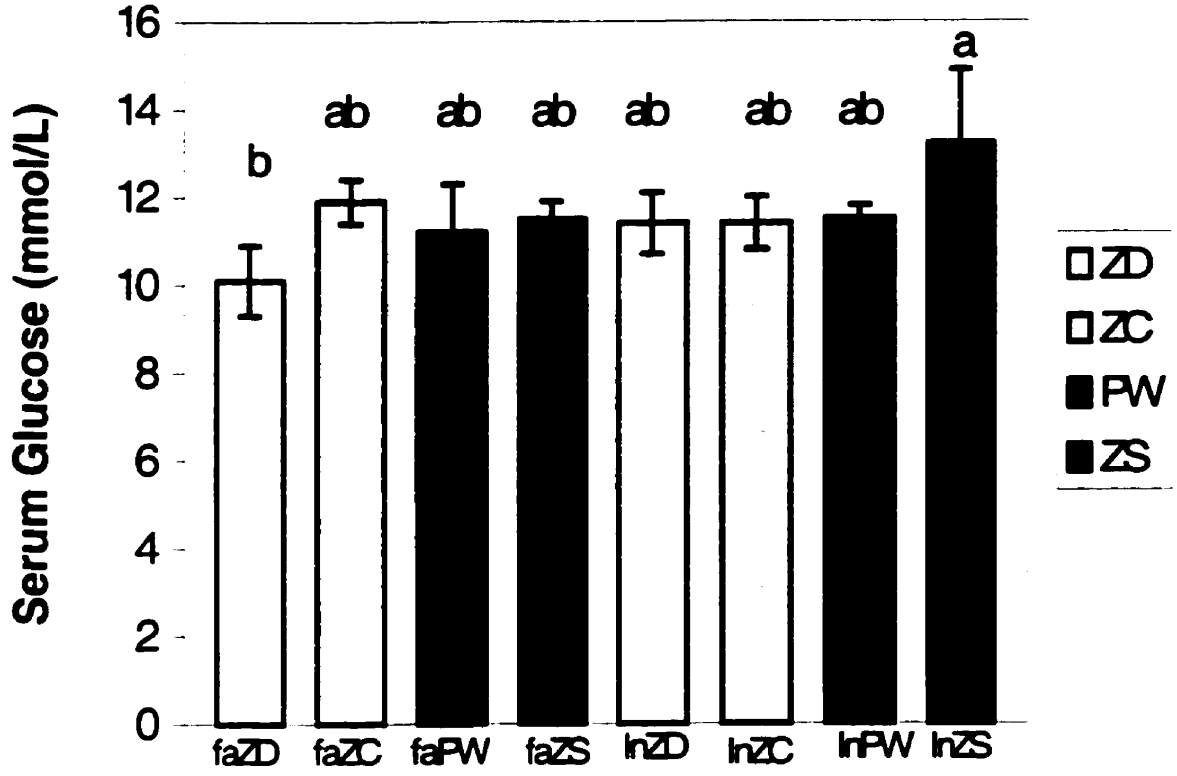


Figure 10. The effects of dietary zinc on serum glucose concentration of obese (fa/fa) and lean Zucker rats after 9 weeks of dietary treatment. Treatment groups were faZD=fa/fa zinc deficient diet, faZC=fa/fa zinc control diet, faPW=fa/fa pair-weighed, faZS=fa/fa zinc supplemented diet, lnZD=lean zinc deficient diet, lnZC=lean zinc control diet, lnPW=lean pair-weighed and lnZS=lean zinc supplemented diet. Each bar represents the mean serum glucose concentration ± SEM for n=7 rats. Different lowercase letters indicate significant differences between means as determined by Duncan's multiple range test.

particularly interesting considering that both groups were provided diets with the same level of dietary zinc (30 ppm). Among the lean dietary treatment groups, there was a gradual increase in creatinine excretion from week 0 to week 9 of the experiment period and there was no effect of diet (Table 15).

The results for urine zinc at 3 week intervals during the dietary intervention experiment are presented in Table 16. Within the fa/fa genotype groups, the faZC and faZS rats did not show significant changes (increase or decrease) in urine zinc as the experiment progressed to week 9. On the contrary, the faZD rats excreted similar amounts of zinc at the end of the experiment as the levels observed at baseline (before dietary zinc intervention) and lower amounts at week 3 and week 6. Furthermore, the faPW (30 ppm zinc) rats excreted significantly more zinc at week 9 compared to all other time points measured, a trend not observed in the faZC rats also fed the 30 ppm zinc diet. The lean rats showed a more similar pattern in urinary zinc excretion among treatment groups. As shown in Table 16, all lean rats, regardless of the level of dietary zinc intervention, demonstrated higher levels of urine zinc at weeks 3, 6 and 9 compared to baseline. In summary, the fa/fa rats showed more variability in urine zinc levels throughout the experiment compared to the lean rats. Of more interest is the observation that neither a ZD, ZC or ZS diet increased zinc excretion in the fa/fa rats whereas pair-weighing increased urine zinc compared to baseline in this obese rat model.

Changes in urine glucose throughout the experiment are shown in Table 17. Within the fa/fa rats, the faZD and faPW rats showed increases in urine glucose excretion at week 9 compared to baseline, whereas urine glucose did not significantly change

during the study in the faZC and faZS groups. Among the lean rats, there were no differences in urine glucose between time points in the lnZD, lnZC and lnZS groups. However, there was increased urine glucose at week 9 compared to baseline in the lnPW rats.

Glucose Transporter Protein 4 (Glut 4) Expression in Adipocytes

Western Immunoblotting was used to evaluate the expression of total Glut 4 in adipocytes of obese and lean Zucker rats after 9 weeks of dietary zinc intervention. Analysis of variance demonstrated a significant genotype and dietary zinc effect on the expression of glut 4 (Appendix A). The fa/fa rats expressed less Glut 4 compared to the lean rats (995 ± 150 vs. 1650 ± 142, arbitrary units, respectively). In terms of dietary zinc, there was less Glut 4 expressed in the ZS treatment group compared to the ZD and ZC groups (886 ± 161, 1518 ± 218 and 1473 ± 172 arbitrary units, respectively). Table 18 and Figure 12 and 13 demonstrate the results for Glut 4 expression among all 8 treatment groups. The faZS group had significantly less expression of Glut 4 compared to all other treatment groups (lean and fa/fa).

Table 13. Effects of dietary zinc on urine volume and urinary zinc, glucose and creatinine excretion of obese (fa/fa) and lean Zucker rats **after 9 weeks** of dietary treatment[1]

	Dietary Treatment Groups[2]							
	fa/fa				**lean**			
	faZD	**faZC**	**faPW**	**faZS**	**lnZD**	**lnZC**	**lnPW**	**lnZS**
Urine Volume (ml/12 hours)	9.42^{a} ± 1.47	8.43^{a} ± 2.05	11.62^{a} ± 1.21	8.18^{a} ± 1.68	9.07^{a} ± 5.34	7.61^{a} ± 2.04	6.60^{a} ± 0.81	5.04^{a} ± 1.14
Urine Creatinine (mg/12 hours)	3.06^{b} ± 0.37	2.94^{b} ± 0.33	2.50^{b} ± 0.40	3.33^{b} ± 0.36	3.76^{b} ± 0.62	5.01^{a} ± 0.61	5.24^{a} ± 0.17	3.17^{b} ± 0.51
Urine Zinc (mg zinc/mg creatinine)	2.40^{bc} ± 0.40	3.29^{bc} ± 0.78	8.01^{a} ± 2.46	4.49^{b} ± 0.84	0.63^{c} ± 0.17	0.60^{c} ± 0.07	0.45^{c} ± 0.09	1.10^{c} ± 0.22
Urine Glucose (mg glucose/mg creatinine)	21.7^{b} ± 13.3	15.2^{b} ± 11.4	76.5^{a} ± 38.1	23.1^{b} ± 15.4	0.09^{b} ± 0.02	0.08^{b} ± 0.02	0.04^{b} ± 0.01	0.20^{b} ± 0.10

[1] Values represent the mean ± SEM for n=7 rats except n=6 for lnZD and n=6 for lnZC urine glucose. Urine zinc and glucose measurements represent values corrected to creatinine excretion. Different superscript letters indicate significant differences between means as determined by Duncan's multiple range test.

[2] fa/fa=obese Zucker rat, lean=lean Zucker rat, faZD=fa/fa zinc deficient diet, faZC=fa/fa zinc control diet, faPW=fa/fa pair-weighed, faZS=fa/fa zinc supplemented diet, lnZD=lean zinc deficient diet, lnZC=lean zinc control diet, lnPW=lean pair-weighed and lnZS=lean zinc supplemented diet.

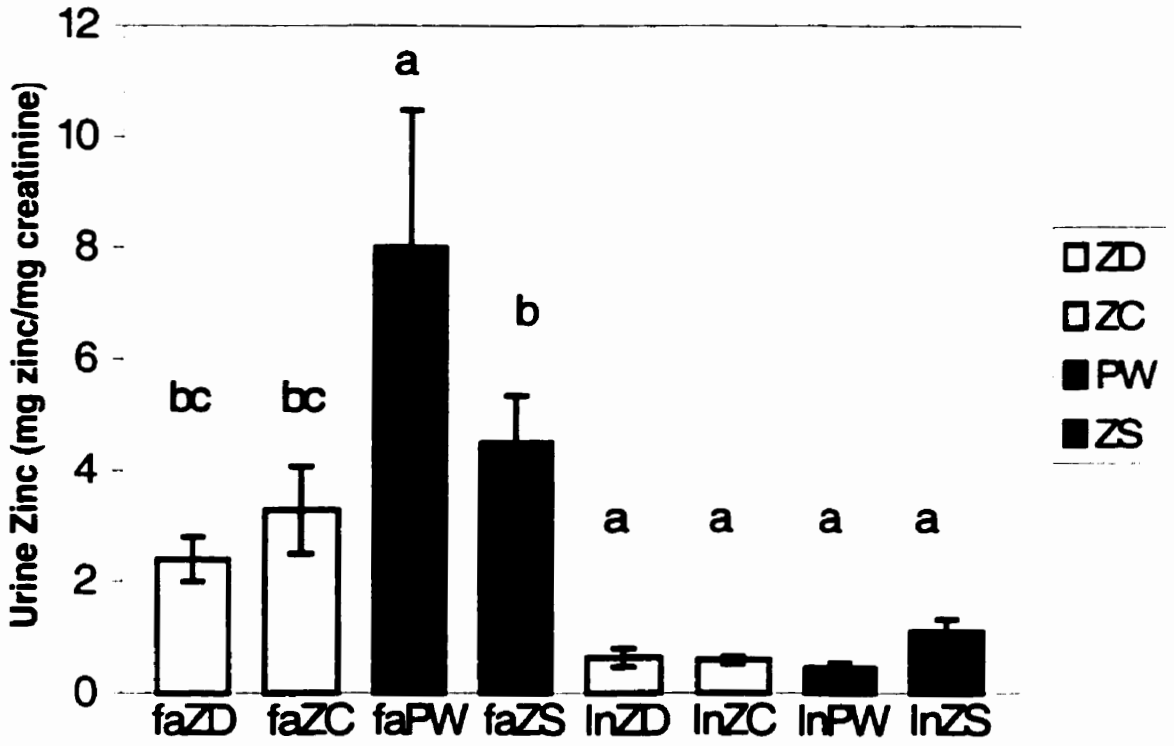


Figure 11. The effects of dietary zinc on urinary zinc excretion of obese (fa/fa) and lean Zucker rats after 9 weeks of dietary treatment. Urine zinc represents values corrected to creatinine excretion. Treatment groups were faZD=fa/fa zinc deficient diet, faZC=fa/fa zinc control diet, faPW=fa/fa pair-weighed, faZS=fa/fa zinc supplemented diet, lnZD=lean zinc deficient diet, lnZC=lean zinc control diet, lnPW=lean pair-weighed and lnZS=lean zinc supplemented diet. Each bar represents the mean urine zinc excretion ± SEM for n=7 rats except n=6 for lnZD. Different lowercase letters indicate significant differences between means as determined by Duncan's multiple range test.

Table 14. Effects of dietary zinc on urine volume of obese (fa/fa) and lean Zucker rats at weeks 0, 3, 6 and 9 of dietary treatment[1]

	Dietary Treatment Groups[2]							
	fa/fa				lean			
	faZD	faZC	faPW	faZS	lnZD	lnZC	lnPW	lnZS
Urine Volume Week 0 (ml)	6.35[a] ±0.64	6.98[a] ±0.93	8.09[b] ±1.19	6.76[a] ±0.86	3.32[a] ±0.91	2.81[b] ±0.57	2.55[b] ±0.30	3.01[a] ±0.62
Urine Volume Week 3 (ml)	8.01[a] ±0.76	9.35[a] ±1.27	8.08[b] ±0.92	7.27[a] ±0.39	4.22[a] ±0.85	5.90[a] ±1.24	5.10[ab] ±0.94	4.91[a] ±1.12
Urine Volume Week 6 (ml)	8.74[a] ±0.95	10.0[a] ± 2.7	9.79[ab] ± 1.14	8.02[a] ±1.30	5.46[a] ±1.30	4.59[ab] ±0.88	5.67[a] ±1.11	6.34[a] ±1.24
Urine Volume Week 9 (ml)	9.42[a] ±1.47	8.43[a] ±2.05	11.6[a] ± 1.2	8.18[a] ±1.68	9.07[a] ±5.34	7.61[ab] ±2.04	6.60[a] ±0.81	5.04[a] ±1.14

[1] Values represent the mean ± SEM for n=7 rats except n=6 for lnZD week 9. Different superscript letters indicate significant differences between **means of different weeks within the same treatment group** as determined by Duncan's multiple range test.

[2] fa/fa=obese Zucker rat, lean=lean Zucker rat, faZD=fa/fa zinc deficient diet, faZC=fa/fa zinc control diet, faPW=fa/fa pair-weighed, faZS=fa/fa zinc supplemented diet, lnZD=lean zinc deficient diet, lnZC=lean zinc control diet, lnPW=lean pair-weighed and lnZS=lean zinc supplemented diet.

Table 15. Effects of dietary zinc on urine creatinine excretion of obese (fa/fa) and lean Zucker rats at weeks 0, 3, 6 and 9 of dietary treatment[1]

	Dietary Treatment Groups[2]							
	fa/fa				lean			
	faZD	faZC	faPW	faZS	lnZD	lnZC	lnPW	lnZS
Urine Creatinine Week 0 (mg/12 hours)	1.55^{c} ± 0.22	1.87^{a} ± 0.33	1.64^{b} ± 0.18	1.22^{c} ± 0.17	1.15^{c} ± 0.12	1.08^{c} ± 0.15	1.09^{c} ± 0.18	1.08^{b} ± 0.10
Urine Creatinine Week 3 (mg/12 hours)	2.30^{b} ± 0.19	2.01^{a} ± 0.12	2.24^{ab} ± 0.21	2.22^{b} ± 0.27	2.57^{b} ± 0.35	3.38^{b} ± 0.16	3.40^{b} ± 0.31	2.99^{a} ± 0.35
Urine Creatinine Week 6 (mg/12 hours)	3.50^{a} ± 0.27	3.03^{a} ± 0.51	3.02^{a} ± 0.37	3.77^{a} ± 0.39	3.81^{a} ± 0.40	3.37^{b} ± 0.52	3.56^{b} ± 0.56	3.96^{a} ± 0.39
Urine Creatinine Week 9 (mg/12 hours)	3.06^{a} ± 0.37	2.94^{a} ± 0.33	2.50^{ab} ± 0.40	3.33^{a} ± 0.36	3.76^{a} ± 0.62	5.01^{a} ± 0.61	5.24^{a} ± 0.17	3.17^{a} ± 0.51

[1] Values represent the mean ± SEM for n=7 rats except n=6 for lnZD at week 9. Different superscript letters indicate significant differences between **means of different weeks within the same treatment group** as determined by Duncan's multiple range test.

[2] fa/fa=obese Zucker rat, lean=lean Zucker rat, faZD=fa/fa zinc deficient diet, faZC=fa/fa zinc control diet, faPW=fa/fa pair-weighed, faZS=fa/fa zinc supplemented diet, lnZD=lean zinc deficient diet, lnZC=lean zinc control diet, lnPW=lean pair-weighed and lnZS=lean zinc supplemented diet.

Table 16. Effects of dietary zinc on urine zinc excretion of obese (fa/fa) and lean Zucker rats at weeks 0, 3, 6 and 9 of dietary treatment[1]

	Dietary Treatment Groups[2]							
	fa/fa				lean			
	faZD	faZC	faPW	faZS	lnZD	lnZC	lnPW	lnZS
Urine Zinc Week 0 (mg zinc/mg creatinine)	2.62[a] ± 0.44	3.62[a] ± 0.73	3.35[b] ± 0.42	3.58[a] ± 0.34	2.00[a] ± 0.24	2.62[a] ± 0.48	2.14[a] ± 0.30	1.87[a] ± 0.28
Urine Zinc Week 3 (mg zinc/mg creatinine)	0.69[b] ± 0.10	3.05[a] ± 0.23	3.01[b] ± 0.40	3.65[a] ± 0.57	0.43[b] ± 0.09	0.62[b] ± 0.10	0.53[b] ± 0.08	1.19[b] ± 0.29
Urine Zinc Week 6 (mg zinc/mg creatinine)	1.21[b] ± 0.16	4.25[a] ± 2.36	3.88[b] ± 0.82	3.63[a] ± 0.74	0.51[b] ± 0.05	0.59[b] ± 0.09	0.63[b] ± 0.09	0.60[b] ± 0.07
Urine Zinc Week 9 (mg zinc/mg creatinine)	2.40[a] ± 0.40	3.29[a] ± 0.78	8.01[a] ± 2.46	4.49[a] ± 0.84	0.63[b] ± 0.17	0.60[b] ± 0.07	0.45[b] ± 0.09	1.10[b] ± 0.22

[1] Values represent the mean ± SEM for n=7 rats except n=6 for lnZD week 9. Urine zinc measurements represent values corrected to creatinine excretion. Different superscript letters indicate significant differences between **means of different weeks within the same treatment group** as determined by Duncan's multiple range test.

[2] fa/fa=obese Zucker rat, lean=lean Zucker rat, faZD=fa/fa zinc deficient diet, faZC=fa/fa zinc control diet, faPW=fa/fa pair-weighed, faZS=fa/fa zinc supplemented diet, lnZD=lean zinc deficient diet, lnZC=lean zinc control diet, lnPW=lean pair-weighed and lnZS=lean zinc supplemented diet.

Table 17. Effects of dietary zinc on urine glucose excretion of obese (fa/fa) and lean Zucker rats at weeks 0, 3, 6 and 9 of dietary treatment[1]

	Dietary Treatment Groups[2]							
	fa/fa				**lean**			
	faZD	**faZC**	**faPW**	**faZS**	**lnZD**	**lnZC**	**lnPW**	**lnZS**
Urine Glucose Week 0 (mg glucose/mg creatinine)	0.13^{b} ± 0.02	0.14^{a} ± 0.01	0.16^{b} ± 0.02	0.26^{a} ± 0.09	0.11^{a} ± 0.01	0.12^{a} ± 0.02	0.12^{a} ± 0.01	0.12^{a} ± 0.02
Urine Glucose Week 3 (mg glucose/mg creatinine)	0.17^{b} ± 0.05	28.0^{a} ± 27.7	0.24^{b} ± 0.05	0.99^{a} ± 0.46	0.10^{a} ± 0.03	0.04^{b} ± 0.00	0.05^{b} ± 0.01	0.09^{a} ± 0.02
Urine Glucose Week 6 (mg glucose/mg creatinine)	0.45^{b} ± 0.24	149^{a} ± 148	1.96^{b} ± 1.34	1.06^{a} ± 0.49	0.06^{a} ± 0.01	0.06^{b} ± 0.01	0.07^{b} ± 0.02	0.06^{a} ± 0.01
Urine Glucose Week 9 (mg glucose/mg creatinine)	21.7^{a} ± 13.3	15.2^{a} ± 11.4	76.5^{a} ± 38.1	23.1^{a} ± 15.4	0.09^{a} ± 0.02	0.08^{ab} ± 0.02	0.04^{b} ± 0.01	0.20^{a} ± 0.10

[1] Values represent the mean ± SEM for n=7 rats except n=6 for lnZD and lnZC at week 9. Urine glucose measurements represent values corrected to creatinine excretion. Different superscript letters indicate significant differences between **means of different weeks within the same treatment group** as determined by Duncan's multiple range test.

[2] fa/fa=obese Zucker rat, lean=lean Zucker rat, faZD=fa/fa zinc deficient diet, faZC=fa/fa zinc control diet, faPW=fa/fa pair-weighed, faZS=fa/fa zinc supplemented diet, lnZD=lean zinc deficient diet, lnZC=lean zinc control diet, lnPW=lean pair-weighed and lnZS=lean zinc supplemented diet.

Table 18. Effects of dietary zinc on adipocyte total Glut 4 of obese (fa/fa) and lean Zucker rats after 9 weeks of dietary treatment.

	Dietary Treatment Groups[2]							
	fa/fa				lean			
	faZD	faZC	faPW	faZS	lnZD	lnZC	lnPW	lnZS
Glut 4 (arbitrary units)	1260[a] ±322	1323[a] ±361	938[a] ±105	442[b] ±101	1777[a] ±280	1776[a] ±405	1714[a] ±255	1330[a] ±160

[1] Values represent the mean ± SEM for n=6 rats except n=4 for faPW and n=5 for lnPW. Different superscript letters indicate significant differences between means as determined by Duncan's multiple range test.

[2] fa/fa=obese Zucker rat, lean=lean Zucker rat, faZD=fa/fa zinc deficient diet, faZC=fa/fa zinc control diet, faPW=fa/fa pair-weighed, faZS=fa/fa zinc supplemented diet, lnZD=lean zinc deficient diet, lnZC=lean zinc control diet, lnPW=lean pair-weighed and lnZS=lean zinc supplemented diet.

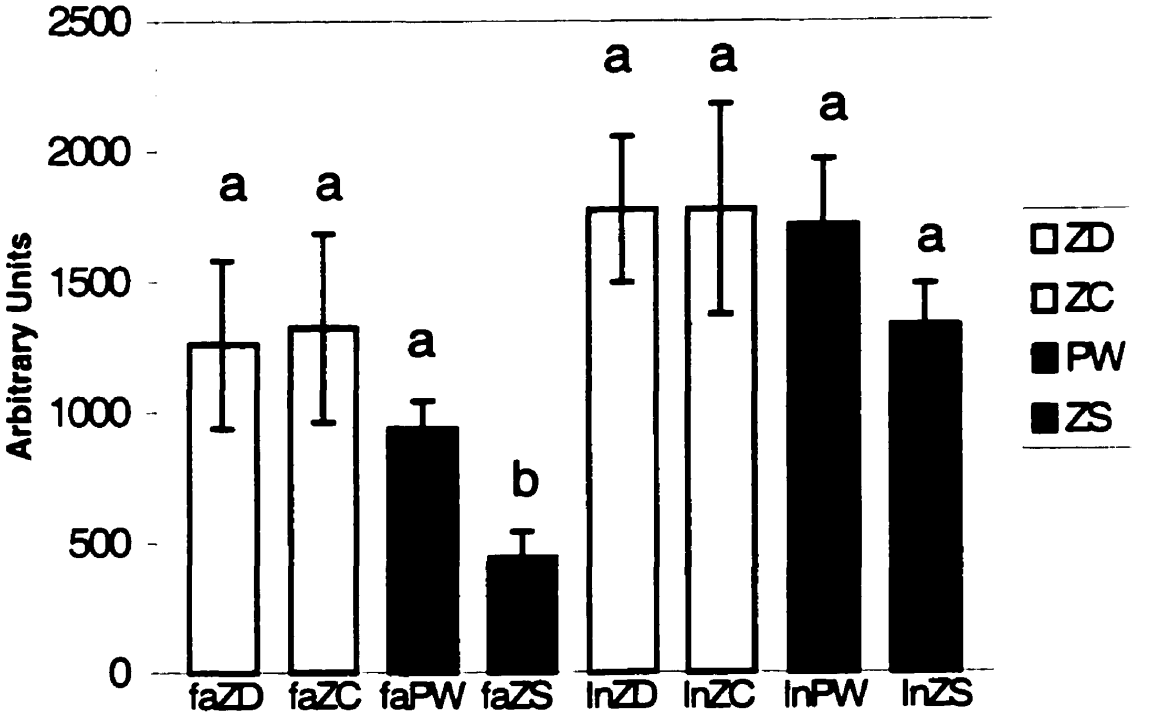


Figure 12. The effect of dietary zinc on the expression of adipocyte total Glut 4 in obese (fa/fa) and lean Zucker rats after 9 weeks of dietary treatment. Treatment groups were faZD=fa/fa zinc deficient diet, faZC=zinc control diet, faPW=fa/fa pair-weighed, faZS=fa/fa zinc supplemented diet, lnZD=lean zinc deficient diet, lnZC=lean zinc control diet, lnPW=lean pair-weighed and lnZS=lean zinc supplemented diet. Each bar represents the mean adipocyte Glut 4 ± SEM for n=6 rats except n=4 for faPW and n=5 for lnPW. Different lowercase letters indicate significant differences between means as determined by Duncan's multiple range test.

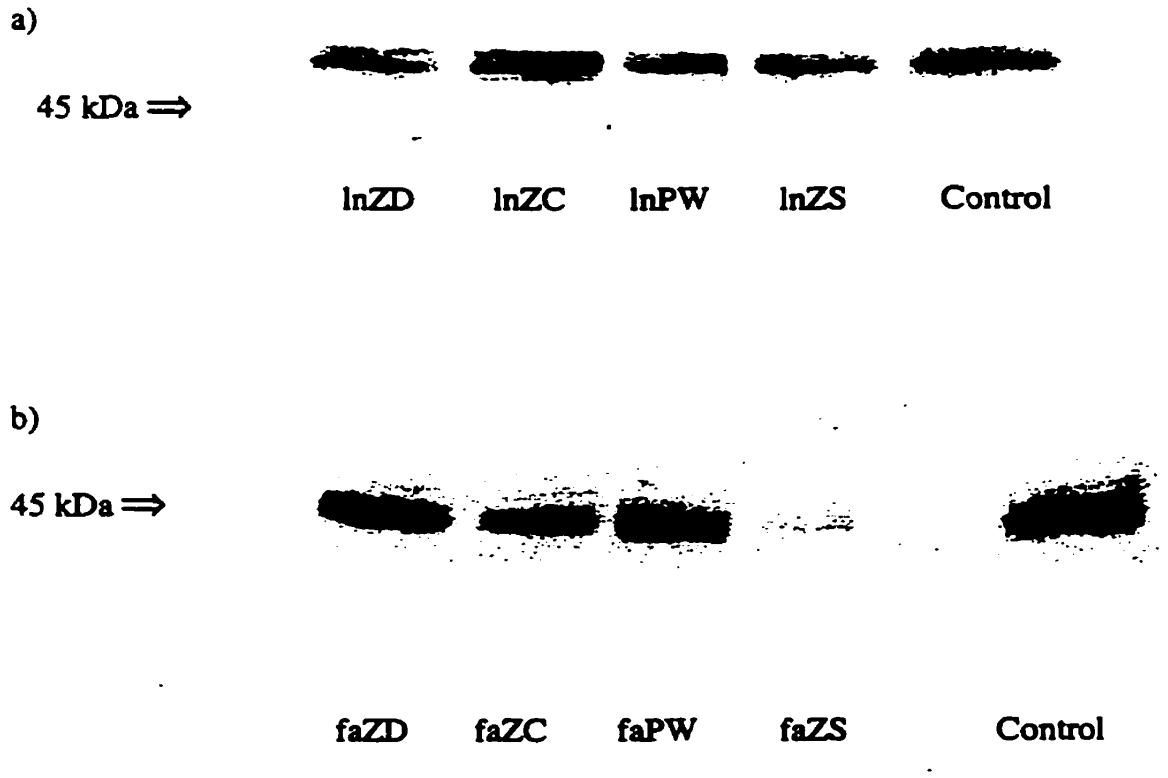


Figure 13. The effect of dietary zinc on the expression of adipocyte total Glut 4 in obese (fa/fa) and lean Zucker rats after 9 weeks of dietary treatment. Treatment groups were faZD=fa/fa zinc deficient diet, faZC=zinc control diet, faPW=fa/fa pair-weighed, faZS=fa/fa zinc supplemented diet, lnZD=lean zinc deficient diet, lnZC=lean zinc control diet, lnPW=lean pair-weighed and lnZS=lean zinc supplemented diet. The expression of Glut 4 in a) lean adipocytes and b) fa/fa adipocytes was detected by Western immunoblotting (procedure described in Methods section).

V. Discussion

There are several main findings in this thesis project. Firstly, the fa/fa rats expressed less Glut 4 than their lean littermates, and diet (zinc supplementation) combined with genetics (fa/fa) further reduced adipocyte Glut 4 expression in Zucker rats (Table 18). Based on our results, there are indications that zinc supplementation does *not* aggravate body or hepatic fat deposition or adipocyte size in the Zucker rat (Table 4 and Table 9). Furthermore, despite the role of zinc in insulin action, the results of this study suggest that dietary zinc supplementation (150 ppm) does not attenuate hyperinsulinemia in the fa/fa rat (Table 12). In terms of zinc status, it appears that the fa/fa rats have higher femur and serum zinc concentrations but lower hepatic zinc stores compared to the lean rats. Interestingly, a zinc supplemented diet elevated serum zinc concentrations in lean rats but this effect was not observed in the fa/fa genotype. Each of these results, together with results for other topic areas investigated in this project, will be discussed in detail in the following sections.

Feed Intake

The obese (fa/fa) Zucker rat is characterized by increased food intake (hyperphagia) which contributes to the development of obesity in this animal model (White and Martin, 1997). In our study, analysis of total feed intake for 9 weeks revealed that fa/fa rats consumed 26.3% more grams of diet compared to lean rats. This difference translated to approximately 8 grams more diet consumed per day by the fa/fa rats. Our results for feed intake are consistent with Ionescu et al. (1985) who demonstrated that 13 to 14 week old fa/fa Zucker rats consumed 31.3 grams diet per day compared to 22.9 grams of diet consumption by lean Zucker rats.

Based on White and Martin (1997), hyperphagia in the fa/fa rat is linked to a disrupted feeding mechanism in the brain, specifically, a defect in the leptin signaling pathway. Leptin is a hormone produced and released from the adipose tissue that transmits a satiety signal to the central nervous system (Takahashi et al., 1996). This satiety signal occurs via leptin's ability to *inhibit* neuropeptide Y (NPY), a potent inducer of food intake (White and Martin, 1997). Interestingly, the fa/fa rat is characterized by a genetic defect in the leptin receptor resulting in "leptin resistance" (Bray and York, 1997). Therefore, the fa/fa rat is resistant to the action of leptin, resulting in elevated NPY levels that may ultimately contribute to the increased feed intake of these animals. Although we did not analyze for serum leptin or NPY concentrations, an understanding of the role of leptin and NPY, and the evidence that the fa/fa rat is leptin resistance, provides an explanation for the increased feed intake observed in our study and in the literature.

As shown in Table 3 and Figure 1, there were no differences in feed intake among the dietary treatment groups. We did not observe decreased feed intake (anorexia) in the rats fed a zinc deficient diet compared to the rats fed a zinc control or zinc supplemented diet. Rodents fed a zinc deficient diet (< 1 ppm zinc) commonly show signs of zinc deficiency, that is, the development of anorexia and hence a reduction in feed intake (Prasad, 1983). In our experiment, we used a 5 ppm level of zinc for our ZD diet to induce a "marginal" zinc deficiency. The use of a marginal zinc deficient diet may explain why the rats did not show one of the clinical signs of zinc deficiency (anorexia/reduced feed intake).

Body Weight

The fa/fa rat is a model of obesity and therefore it was not surprising to find a significantly higher body weight in the fa/fa compared to the lean rats (Table 4). The obese condition is clearly visible at 5 weeks of age, as cited in the literature (Johnson et al., 1971). In our study, a significant difference in weight was observed at 5 weeks of age between genotype groups and this difference was maintained throughout the study.

Although growth retardation, secondary to reduced feed intake, is another common feature of zinc deficiency (Aggett and Comerford, 1995), we did not observe any differences between the rats fed diets containing different levels of zinc. Again, it is assumed that a restriction of 5 ppm zinc was not sufficient to induce growth retardation.

We were successful in maintaining the PW rats at similar body weights as the rats in the zinc deficient group. Should we have seen significant decreases in weight of the ZD rats compared to the other treatment groups, we would not have been able to attribute our findings to the diet.

Body Fat

The significant differences in body weight between genotype groups can be attributed to more body fat in the fa/fa rat compared to lean controls. In our experiment, we measured epididymal and perirenal fat pad weights that are representative of abdominal obesity (Ishida et al., 1996). The fa/fa rats had epididymal and perirenal fat pads that were 65.3% and 75.9% heavier, respectively, compared to the lean rats (Table 4). It appeared that among the fa/fa rats only, the perirenal fat pads were heavier compared to the epididymal fat weights (27.2 ± 0.9 vs. 18.4 ± 0.7 g, respectively). Therefore, our results showed that the perirenal fat pad contributes more to the total fat

pad weight in fa/fa rats. The observations of Richards et al. (2000) who demonstrated similar weights among the epididymal and perirenal fat pad weights of fa/fa rats do not support this finding.

We calculated the relative total fat weight, as we were interested in the percentage of body weight that can be attributed to abdominal obesity. Our results were similar to that of Johnson et al. (1971) for the Zucker rat. The fa/fa rat has a larger percentage of weight attributed to abdominal fat depots (Appendix A and Table 4).

According to Chen et al. (1996), zinc supplementation aggravates body fat deposition in the obese (ob/ob) mouse, implying that zinc supplementation may be detrimental to the development of obesity. However, dietary zinc supplementation did not influence fat deposition in our study. In both genotype groups, the zinc-supplemented rats had similar fat pad weights as the zinc deficient and zinc control dietary treatment groups (Table 4).

There are several methodological differences that may explain the differences between our study and that of Chen et al. (1996). Firstly, Chen et al. (1996) demonstrated that ob/ob mice fed a zinc supplemented diet showed increased *total carcass body fat* content compared to mice fed a diet with marginal zinc. Compared to our experiment, in which we measured the epididymal and perirenal fat weights as measures of body fat deposition, Chen et al. (1996) measured the effects of zinc on total carcass fat content. We did not measure total carcass fat and therefore can not report what effect our dietary intervention had on this parameter. Our decision to measure fat pad weights (versus total carcass fat) stems from the understanding that abdominal obesity is a key risk factor to insulin resistance and Type 2 diabetes (Wahlqvist et al.,

1999). We were not interested in simple total body fatness but more the distribution of adipose tissue and what effect (if any) zinc supplementation had on abdominal obesity.

Our study and that of Chen et al. (1996) also differed in the animal model used. Whereas both studies used animal models of obesity (the Zucker rat and the ob/ob mouse), the models do have important physiological differences. The ob/ob mouse is a leptin *deficient* animal model, compared to the leptin *resistant* fa/fa rat (Bray & York, 1997). It is unclear what effect leptin deficiency or leptin resistance has on influencing the interaction of zinc and body fat deposition. To our knowledge, the fat pad weights have not been measured in the fa/fa Zucker rat after dietary zinc interventions.

The fa/fa rat is similar to the db/db mouse model, both rodents being leptin resistant due to mutations in the leptin receptors (Caro et al., 1996). The db/db mouse has been studied in a dietary zinc manipulation study (Simon, 1998). However, similar to our results for the fa/fa rats, Simon (1998) did not demonstrate that zinc supplementation increased epididymal fat pad weight compared to mice fed a zinc control diet.

Therefore, we showed that zinc supplementation (150 ppm for 9 weeks) was not detrimental on increasing abdominal obesity in fa/fa or lean rats. Interestingly, our results for body fat did show a significant genotype x zinc interaction (Appendix A) on the *relative* epididymal fat weight of the fa/fa rats. This genotype x zinc interaction was demonstrated in the faPW rats which had significantly higher (16%) *relative* epididymal fat weights compared to the faZS rats (3.24 ± 0.20 vs. 2.70 ± 0.11 g/100 g body weight, respectively). The results of our study suggest that there is an interaction of zinc, genotype and the feeding pattern of fa/fa rats.

Pair-weighed (or pair fed) animals have similar feeding patterns as meal-fed rats and meal-feeding has been shown to enhance glucose utilization in the adipose tissue of rats (Reeves and O'Dell, 1983). Reeves and O'Dell demonstrated that Wistar rats fed a zinc control (100 ppm) diet *ad libitum* for 3 weeks had heavier epididymal fat pad weights compared to meal-fed rats fed the same diet. However, the meal fed rats demonstrated increased (120%) conversion of ^{14}C to fatty acids in the epididymal fat depot. Although the fat pads were heavier in the *ad libitum* group after 3 weeks, the conversion of glucose to fat was actually higher in the meal-fed rats. Our study was extended to 9 weeks and perhaps at 9 weeks, the increased lipogenesis observed in meal-fed animals at 3 weeks, ultimately manifests itself as increased body fat. Although the Wistar rat is not an animal model of obesity, the results of Reeves and O'Dell (1983) lend insight on the metabolic changes observed in meal-fed rats. The results of these authors may in part explain the significant genotype x zinc interaction observed on the relative epididymal fat pad weight of the faPW rats in our experiment.

Adipocyte Diameter

The heavier fat pad weights of the fa/fa rats may be explained by larger adipocytes (fat cells). In this experiment, the fa/fa adipocytes had diameters that were 19.7% larger than adipocytes from lean rats (Appendix A). Similar to our result for fat pad weights, there was no effect of dietary zinc on the diameters of the adipocytes (Table 4). Therefore, our results for adipocyte diameters are another indication that zinc supplementation (150 ppm) did not aggravate body fat (adipocyte size) in this obese rat model. To our knowledge, this is the first study that investigated the effects of dietary zinc on adipocyte diameters.

Adipocyte diameters are not routinely measured in the Zucker rat although it is a technique used in literature for other rodent models (Parrish et al., 1991). An alternative means of determining adipocyte size is by calculating the amount of lipid per cell (μg lipid per cell) as demonstrated by Johnson et al. (1971) for the Zucker rat. The results of Johnson et al. (1971) confirm our observation of larger adipocyte in the 15 week old fa/fa rat compared to the lean controls.

The epididymal fat pad is commonly used for the isolation of adipocytes from Zucker rats (Pederson et al., 1992; Guerre-Millo et al., 1985; Czech et al., 1978). Our results showed that dietary zinc did not influence the size of the fat cells isolated from this particular fat depot. A limitation of our work is that we did not isolate adipocytes from the perirenal fat pad. Perirenal fat pads are known to be more responsive to dietary manipulation (Parrish et al., 1991) but the effect (if any) that dietary zinc had on the adipocytes of this fat pad can not be elucidated from our work. However, it is noteworthy that in our experiment, dietary zinc did not influence the perirenal fat pad weights in lean and fa/fa rats fed a 150 ppm zinc supplemented diet for 9 weeks (Table 4).

Organ Weights: Kidney, Small Intestine, Spleen and Pancreas

Studies have shown a positive correlation between kidney size and glomerular filtration rate as well as renal plasma flow (Koh et al., 1985) and kidneys in diabetic rats have been shown to be enlarged. In our study, the average weight for both kidneys was 4.1 $\pm$ 0.1 grams for the fa/fa rats (approximately 2.0 grams per kidney) compared to 3.0 $\pm$ 0.0 grams in the lean rats (approximately 1.5 grams per kidney), a difference that reached statistical significance (Appendix B and Table 7). Our results were different from Koh et

al. (1985) and Ward et al. (1994). These authors did not observe significant differences in kidney weight between the fa/fa and lean rats. Koh et al. (1985) reported kidney weights of 1.00 ± 0.03 grams/kidney for both the fa/fa and lean rats, and Ward et al. (1994) reported kidney weights of 1.14 ± 0.04 grams/kidney and 1.04 ± 0.06 grams/kidney in the fa/fa and lean rats, respectively. Therefore, we not only showed significant differences in kidney weights between genotypes (a difference not supported by the literature cited), but the kidney weights in general were heavier in our experiment, particularly in the fa/fa rats. A possible explanation for the higher kidney weights observed in our study was the heavier body weights of the rats in our study compared to age-matched fa/fa rats used by Koh et al. (1985) and Ward et al. (1994). Therefore, we used the relative kidney weight calculation to take into account differences in body weight, given that there was a significant genotype effect on body weights. The relative kidney weights of the fa/fa rats were actually lower than the lean rats, a finding supported by the literature (Koh et al., 1985; Ward et al., 1994). An interesting observation is the higher relative kidney weights in the faPW rats compared to the faZC rats receiving the same level of dietary zinc. The larger kidneys may be a reflection of deteriorating kidney function, also reflected in the increases of urine glucose and zinc (Table 13).

To our knowledge, the weight and length of the small intestine of the Zucker rat has not been reported in the literature. In the ob/ob mouse, small intestine length and weight do not differ between the ob/ob and lean genotypes (Kennedy and Failla, 1987). The purpose of reporting small intestine length and weight was to use these parameters as another measure of growth in the Zucker rat following dietary zinc manipulation. Similar to our results for body weight, dietary zinc did not influence small intestine weight or

length (Table 7). We did however show significant genotype variability where the fa/fa rats had heavier and longer small intestine compared to lean rats. The heavier small intestine in the obese rat may have been a reflection of the higher body weights since the relative small intestine weights were actually smaller in the fa/fa rats compared to the lean rats. Small intestine weight and length also lend insight on the absorptive properties of the Zucker rat. We have demonstrated an improved zinc status in the fa/fa rat compared to lean rats, despite increases in urine zinc excretion (*See Discussion sections on femur, liver and serum zinc, and urinary parameters*). Interestingly, we observed significantly longer and heavier small intestines in the obese rat model, implying that the absorptive area of the small intestine is increased in the fa/fa rats and this may possibly affect (increase) the absorption of zinc from the diet.

The larger spleens observed in the fa/fa rat compared to the lean controls appeared to be a function of heavier body weights (Table 4). Although the weight of the spleens showed genotypic variability, calculation of the relative spleen weights revealed smaller spleen weights in the fa/fa rats when expressed per 100 g body weight. Zinc per se did not affect spleen weight or relative spleen weight (Table 8) and to our knowledge, spleen weights for Zucker rats after dietary zinc manipulation have not been documented in the literature.

We reported significantly higher pancreatic weights in the lean rats compared to the fa/fa rat (Appendix B and Table 8). This is supported in the literature by Schneeman et al. (1983) whereas Meereis-Schwanke et al. (1998) did not show a significant genotype difference. We did not observe a significant zinc effect on the weight of the pancreas in either the lean or fa/fa groups. To our knowledge, the effect of zinc on the weight of the

pancreas for Zucker rats has not been reported in the literature. We did not do further analysis on the pancreas of the Zucker rats. It would be interesting to study the effects of zinc supplementation on the size of the pancreatic β-cells.

Liver Weight and Liver Lipid

Liver weights in this study showed a significant genotype effect (Appendix C). The fa/fa rats had significantly heavier livers compared to the lean rats, an observation that is supported by other investigations with this animal model (Serfass et al., 1988; Koh et al., 1985). Since the body weights were also significantly higher in the fa/fa rats, calculation of the relative liver weights was necessary, although this measurement is not always reported in the literature. The results for relative liver weight confirmed that the livers of fa/fa rats were heavier than the lean rats when the differences in body weight were considered.

In our experiment, zinc did not influence liver weights of either the fa/fa or lean rats (Appendix C). Conversely, Cunnane (1988) found that Sprague-Dawley rats fed a zinc deficient diet (3.5 ppm) had significantly smaller liver weights compared to rats fed a zinc control (36 ppm) or a zinc supplemented diet (411 ppm). Although both our experiment and that of Cunnane (1998) employed similar levels of dietary zinc (zinc deficient and zinc control diets) for equal periods of time, the Sprague-Dawley is not an animal model of obesity and therefore the results can not be directly compared to our experiment.

The larger livers observed in the fa/fa rats were probably a consequence of increased liver lipid. Calculation of both liver lipid concentration and total liver lipid content demonstrated fatty livers in the fa/fa rats compared to the lean groups (Table 9).

The observation of fatty livers in the fa/fa rat is in agreement with the findings of others (Koh et al., 1985; Serfass et al., 1988).

We considered it important to express the liver lipid relative to liver weight, since there was a significant genotype effect on liver weights. Nevertheless, even when the liver weights are accounted for, the fa/fa rats maintained significantly more liver lipid compared to the lean rats.

Similar to our results for fat pad weights, we did not demonstrate a significant effect of zinc on the liver lipid of the fa/fa rats (Table 9). Interestingly, the faPW rats had the highest liver lipid concentrations and total liver lipid content. At first we considered that there may have been an association between the higher serum insulin concentrations observed in this group and the higher liver lipid concentration and total liver lipid content. It was speculated that the higher insulin concentrations were acting to promote increased lipogenesis and lipid storage in the faPW liver. However, the faPW rats also had significantly heavier liver weights (Table 9) compared to the other fa/fa groups and thus, when we calculated the *relative* liver lipid content for the faPW rats, it was in fact similar to the faZD, faZC and faZS rats. Therefore, in regards to our hypothesis of zinc supplementation increasing body fat deposition in the fa/fa rat, this was not the case for the fat pads, adipocyte diameters or liver lipid.

Furthermore, we did not demonstrate a significant effect of zinc on the liver lipid of lean Zucker rats. However, Eder and Kirchgessner (1996) showed that Sprague-Dawley rats fed a zinc *deficient* diet (0.5 ppm) had markedly *increased* concentrations of total lipids in the liver compared to rats fed a zinc adequate (40 ppm) zinc. The use of a 0.5 ppm zinc deficient diet is a severe zinc restriction compared to the marginal (5 ppm)

zinc restriction used in our experiment and may in part explain the differences between our results and those of Eder and Kirchgessner (1996).

There may be other factors that contribute to the fatty livers of the fa/fa rat. In humans with insulin resistance, there are increased amounts of free fatty acids in the serum that are taken up by the liver, esterified into triglycerides and phospholipids and ultimately secreted as very low-density lipoproteins (Bohannon, 1992). The fa/fa rat is a hyperlipidemic animal model (Bray, 1977) and perhaps there is decreased exclusion (secretion) of lipids from the liver, a hypothesis suggested by Eder and Kirchgessner (1996) for the zinc deficient Sprague-Dawley rat. Alternatively, the fatty livers observed among the fa/fa rats may be secondary to less fat being stored in adipose and muscle tissue secondary to insulin resistance, resulting in more adipose being "shuttled" to the liver for storage. The liver lipid profiles, lipoprotein receptors and serum free fatty acid concentrations of fa/fa rats after dietary zinc intervention warrant further investigation to determine the factors contributing to the fatty livers of the fa/fa rats, and what effect (if any) zinc may have on influencing these conditions.

Femur, Liver and Serum Zinc

In our experiment, we used femur, liver and serum zinc measurements to assess the zinc status of the animals. The fa/fa rats had significantly higher (18%) femur zinc concentrations compared to the lean rats (Appendix D and Table 10), an observation supported in the literature for the Zucker rat (Donaldson et al., 1987). As commented in the results section, zinc supplementation did not increase the femur zinc concentration of the fa/fa or lean rats compared to their respective control groups. We can assume that the 150 ppm level of zinc supplementation was not adequate to elevate femur zinc stores.

We can also assume that the rats were therefore not "supplemented" which also provides more insight on the lack of changes observed for basal serum insulin concentrations (Table 12). However, zinc supplementation (150 ppm for 9 weeks) was sufficient to alter total Glut 4 expression in adipocytes isolated from fa/fa rats (Table 18, Figure 12 and Figure 13). According to our knowledge, a zinc supplementation study has not been conducted on the fa/fa rat for comparison to our work.

In contrast to the results for femur zinc, the fa/fa rats had a significantly lower (49.4%) liver zinc concentration, a finding also supported by Donaldson et al. (1987) for the fa/fa rat and by Kennedy et al. (1986) for the ob/ob mouse. The lower hepatic zinc concentrations in the obese animals may be secondary to the greater contribution of liver lipid to the hepatic dry weights. Although the hepatic zinc concentrations were significantly lower in the fa/fa rats, the livers were 2.6 times heavier and contained 285 micrograms more zinc than the lean livers. This finding parallels hepatic zinc analysis of the ob/ob mouse (Kennedy and Failla, 1987). Interestingly, dietary zinc had no effect on liver zinc concentration in either the obese or the lean rats. Perhaps the liver stores a fixed amount of zinc in the liver, regardless of the availability through the diet.

The results for serum zinc showed that fa/fa rats fed a zinc deficient diet (5 ppm) had similar serum zinc concentrations as those fed a zinc supplemented diet (150 ppm). As previously discussed, it is possible that the level of zinc supplementation was not sufficient to *elevate* serum zinc concentrations. Alternatively, a 5 ppm marginal zinc deficient diet was not sufficient to *lower* serum zinc concentrations in the fa/fa rat. This abnormal zinc metabolism was not true for the lean rats that showed an expected pattern of lower femur and serum zinc concentrations in rats fed a zinc deficient diet. It would

be of interest to measure intestinal metallothionein or the activity of 5'-nucleotidase, a zinc dependent enzyme, to further assess zinc status of the rats.

To our knowledge, there have been no dietary zinc intervention studies conducted using the fa/fa rat and therefore at this stage of our research, we have no literature to which we can compare our work. We expected suboptimal zinc status to be observed in the fa/fa rat compared to the lean rats based on the literature for humans. In humans, zinc concentrations in the blood are most commonly used to reflect zinc status. Obese patients show significantly lower (20.9-23.9%) serum zinc concentrations compared to lean controls (Di Martino et al., 1993). Furthermore, Kumar and Rao (1974) showed that non-obese patients with diabetes demonstrated significantly lower plasma zinc compared to control subjects (83.7 ± 5.62 vs. 123.2 ± 2.19 *u*g/100 ml, respectively). However, as demonstrated in our study for the Zucker rat, femur (long term zinc status) and serum (short term zinc status) zinc were both higher in this obese model. Although these findings were unexpected, our results are supported by Donaldson et al. (1987) for the fa/fa Zucker rat and by Kennedy and Failla (1987) for the ob/ob mouse. Thus it appears that suboptimal zinc status is demonstrated in some patient population groups of obesity and/type 2 diabetes, whereas this is not necessarily the observation in animal models of the same disease.

Liver Copper

Diets containing excessive amounts of zinc have been shown to impair the copper status of animals (Shils, Olson & Shike, 1994). Increased amounts of zinc induce the synthesis of intestinal metallothionein that can sequester copper and hence decrease its availability, absorption and storage (liver). Diets containing excessive amounts of zinc

(120-240 ppm) have been shown to impair the copper status of rodents, as shown by biochemical signs (activities of cupro-zinc superoxide dismutase and cytochrome *c* oxidase enzymes) of copper deficiency (L'Abbe & Fisher, 1984). Liver copper concentrations can also be used as a measure of copper status and were analyzed in this experiment to assess the toxicity (or lack thereof) of the zinc supplemented diet used in this study. We did not expect copper deficiency due to the zinc supplemented diet. This assumption was based on our results for femur and serum zinc concentrations (Table 10) in which zinc supplementation did not elevated femur and serum zinc concentrations in lean and fa/fa rats (compared to rats fed a zinc control diet). As expected, there was no significant main effect of zinc on liver copper concentrations (Appendix D and Table 11). Interestingly, we observed a significant genotype effect. Similar to our results for liver zinc concentrations, the fa/fa rats had significantly lower liver copper concentrations (Appendix D) than the lean rats and no differences were observed among dietary treatment groups (Table 11). Our results for liver copper concentrations are supported by Donaldson et al. (1987) for 15 week old lean and obese Zucker rats. The lower liver copper concentrations observed in the obese rats may have been due, in part, to a greater contribution of lipid to the dry liver weight (Table 9). Therefore, based on our results for liver copper concentrations, we can conclude that dietary zinc supplementation of 150 ppm does not significantly affect liver copper concentrations, a marker for copper deficiency/zinc toxicity.

Femur Calcium and Phosphorus

The dry weights of the femurs showed genotype variability (Appendix D and Table 10). In addition, the fa/fa rats had significantly shorter femurs compared to the

lean rats (Appendix D and Table 10). As previously discussed, the femur zinc concentrations were greater in the fa/fa rats compared to the lean rats. To further assess bone mineral content, we analyzed for femur calcium and femur phosphorus concentrations. In addition to higher femur zinc concentrations, the fa/fa rats also had significantly higher femur calcium and phosphorus concentrations compared to the lean rats (Appendix D and Table 11). Femur calcium and phosphorus concentrations have not been documented for the Zucker rat. Of particular interest in our experiment was the observation of *lower* femur zinc concentration in rats (lean and fa/fa) fed a zinc deficient diet in but significantly *higher* femur calcium and phosphorus concentrations in the ZD rats, compared to the ZC, PW and ZS dietary treatment groups. Perhaps the rats fed a zinc deficient diet mobilize less zinc from the bone (to preserve stores under dietary deficiency). This in turn may be positively influencing the femur calcium and phosphorus concentrations.

Serum Insulin and Glucose

Fasting serum insulin and glucose concentrations aid in understanding the degree of insulin resistance associated with obesity. The fa/fa obese rat has been extensively characterized as hyperinsulinemic but euglycemic (Clark et al., 1983). In our study, we demonstrated a significantly higher (22 fold) serum insulin concentration in the fa/fa rats compared to the lean rats (Appendix E, Table 12 and Figure 9). This is a general genotype difference supported by others in the literature for the Zucker rat (Clark et al., 1983; Bray, 1977; Richards et al., 2000).

One of the objectives of our study was to examine the effects of dietary zinc supplementation on the hyperinsulinemia of the fa/fa rat. Zinc is associated with

enhancing the action of insulin (Arquilla et al., 1978; Coulston & Dandona, 1980; Penicaud et al., 1987) and a zinc supplemented diet has been demonstrated to lower basal insulin concentrations of ob/ob mice (Begin-Heick et al., 1985) and db/db mice (Simon, 1998). Based on the documented relationships between zinc and insulin, we hypothesized that the fa/fa rats fed a zinc supplemented diet would have lower serum insulin concentrations compared to rats fed the zinc deficient diet. Conversely, we did not show a significant main effect of zinc on insulin concentrations of the fa/fa *or* lean rats (Appendix E and Table 12). Although the faZS rats had lower serum insulin concentration compared to the faZD rats (Table 12), this difference did not reach statistical significance.

Our results are therefore contradictory to those of others who showed differences in serum insulin concentrations with dietary zinc intervention (Begin-Heick et al., 1985; Simon, 1998). It may be that the Begin-Heick et al. (1995) used a higher level of zinc supplementation (964 ppm vs. 150 ppm, respectively), and Simon (1998) used a different animal model (db/db mouse) compared to our study that may, in part, explain the significant changes in serum insulin observed by these authors.

Interestingly, the faPW rats in our study rats had significantly higher (46.3%) serum insulin concentrations compared to the faZC rats, despite both groups of rats consuming the zinc control diet (Table 12). However, as previously discussed, the feeding patterns of the faPW and faZC rats were quite different and may have contributed to the differences we observed in serum insulin concentrations. The pair-weighed rats were essentially meal-feeders and consumed a given amount of diet within a short period of time (approximately 3 hours), followed by "fasting" until diet was provided again the

following day (refer to Results Section: Feed Intake). We speculated that the eating behavior of the faPW rats may have elevated serum insulin concentrations. On the contrary to our findings for the faPW rats, Clark et al. (1983) found that restricting the feed intake of year-old fa/fa rats to 15 grams per day resulted in *decreased* basal serum insulin concentrations, compared to rats given free access to the same diet. It is noteworthy that although Clark et al. (1983) employed the fa/fa Zucker rat (similar to our experiment) the ages of the rat were quite different from our work (52 weeks vs. 15 weeks, respectively). In addition, the length of time that Clark et al. (1983) restricted the feed intake of the fa/fa rats is not clearly indicated, but the level of feed restriction was greater than the present study (15 g/day vs. 28-35 g/day, respectively).

Although the fa/fa rat does not demonstrate hyperglycemia, we were still interested in citing any changes in serum glucose concentration. Begin-Heick et al. (1985) showed that in addition to lowering hyperinsulinemia in dietary zinc supplemented ob/ob mice, basal plasma glucose concentrations were also improved. Simon (1998) demonstrated that dietary zinc supplementation (300 ppm) significantly lowered serum glucose concentrations in db/db mice compared mice fed a zinc deficient (3 ppm) diet for 6 weeks. Furthermore, Chen et al., (1998) verified that zinc supplementation (addition of 20 mM $ZnCl_2$ in the drinking water) of ob/ob mice for 8 weeks significantly reduced plasma glucose concentrations compared to mice provided deionized drinking water. In light of the literature documenting the positive effects of zinc on glycemic control, our study did not show any significant effect due to dietary zinc. The serum glucose concentrations were similar among all the rats, regardless of genotype and dietary zinc treatment. As mentioned, the fa/fa rat is a euglycemic model

compared to the hyperglycemic db/db and ob/ob mouse. Differences in animal models (hyperglycemia vs. euglycemia) explain in part the variations in results observed between our study and those cited in the literature.

Based on our observation of higher insulin concentrations in the faPW compared to the faZC rats, we expected the higher serum insulin to translate into improved glucose uptake by peripheral tissues, resulting in lower serum glucose concentrations. However, this physiological response did not occur, as evident in the similar serum glucose concentrations in the faPW and faZC rats (Table 12 and Figure 9). Perhaps the degree of insulin resistance was greater in the faPW rats and the higher insulin concentrations reflect compensatory hyperinsulinemia.

Our experiment assessed basal serum insulin and glucose concentrations, both of which are important parameters in assessing the degree of insulin resistance in the fa/fa rat. A limitation of our study was that we did not assess the glucose tolerance of the fa/fa rats following dietary zinc intervention. A glucose tolerance test would give insight on the insulin secretory response of the animals in response to a given glucose dose. Begin-Heick et al. (1985) showed that the zinc supplementation decreased the insulin secretory response to a glucose load in the ob/ob mice. Interestingly, Ionescu et al. (1985) demonstrated that although the fa/fa rat is a euglycemic model (normal fasting blood glucose), the obese rat has an abnormal glucose tolerance compared to age-matched lean control rats. More importantly, although Ionescu et al. (1985) showed that baseline serum glucose concentrations did not differ between genotypes (as shown in our study), the ingestion of a glucose load was followed by significantly higher hyperglycemia in the

fa/fa rat. Furthermore, this abnormal glucose tolerance deteriorated with age (Ionescu et al., 1985).

At this stage of our research with the fa/fa rat, we have demonstrated that the 150 ppm zinc level used was not sufficient to improve basal hyperinsulinemia. It remains to be investigated if this level of zinc is appropriate to improve the insulin secretory response and glucose tolerance of the fa/fa rats.

Urinary Parameters: Volume, Creatinine, Zinc and Glucose

Increased urine volume (polyuria) is a clinical sign of Type 2 diabetes. At the end of the study, the fa/fa rats did not exhibit polyuria compared to the lean rats (Table 13), a finding documented in the literature Zucker rats (McCaleb and Sredy, 1992). Polyuria in Type 2 diabetes/insulin resistance is normally a consequence of hyperglycemia. The fa/fa rats in our study were not hyperglycemic compared to the lean rats which aids in explaining the similar urine volumes observed among genotypes. The rats that showed increases in urine volume at the end of the study compared to baseline were the pairweighed fa/fa *and* lean rats (Table 14). It is plausible that there is an influence of feeding patterns on urine volume of the faPW, one that was not influenced by dietary zinc per se. To our knowledge, this is the first observation of a trend towards polyuria in pair-weighed Zucker rats during a 9 week dietary intervention.

With the exception of the pair-weighed animals, we did not see a general increase in urine volumes among the rats with advancing age. This observation is supported by Ikeda et al. (1981) who demonstrated similar urine volumes between 8, 12 and 24-week-old fa/fa rats.

Urinary creatinine was employed in this study to act as a stable measurement against which urine glucose and urine zinc could be quantified. We also used changes in creatinine excretion as a representation of renal function in the fa/fa obese rat. Urinary creatinine values showed a genotypic variability. Given that creatinine excretion is proportional to muscle mass (Bowers and Wong, 1980), it was not surprising to observe increased urinary creatinine in the lean rats (Table 13). Ward et al. (1994) also demonstrated that lean Zucker rats excrete more creatinine on a mg/24 hour basis compared to the fa/fa obese rat. It appears that despite a significantly higher body weight, the fa/fa rats may have significantly lower muscle mass. A limitation of our work is that we did not measure body composition but we did demonstrate a higher relative fat mass in the fa/fa rats (Table 4). Urine was analyzed at baseline and at 3-week intervals to determine any trends (increase or decrease) in creatinine. A comparison between 3 week intervals showed that all of the lean rats (regardless of dietary treatment) had greater urine creatinine excretion at week 9 compared to the beginning of the study (Table 15). On the contrary, increases in urinary creatinine among the fa/fa rats were observed solely in the fa/fa rats fed a zinc deficient or zinc supplemented diet. The increases in urine creatinine among the lean rats may be a reflection of increases in muscle mass as a function of age. Within the fa/fa groups, the zinc deficient and supplemented diet may have also influenced the proportion of muscle mass in these animals. A limitation of our experiment is that we did not do further assessments of renal function. A creatinine clearance test in the Zucker rat would provide more insight on the renal function of this obese model after dietary zinc manipulation.

Human subjects and rodents with insulin resistance (obesity and Type 2 Diabetes) have elevated concentrations of zinc in the urine (Sjorgen et al.,1988; Kumar & Rao 1974; Simon, 1998; Levine et al., 1983). Urine collected at the end of this study showed a significant genotype effect, favoring increased urine zinc in the fa/fa rats (Table 13). Among the fa/fa genotype, the highest concentration of urine zinc was observed in the pair-weighed animals (Table 13). Interestingly, urine zinc excretion did not increase as a result of dietary zinc supplementation in either the lean or fa/fa rats (Table 13). Table 16 shows the trend of urine zinc excretion at 3-week intervals. We demonstrated an interesting decrease in urine zinc excretion among the lean rats at week 9 compared to week 3 of the study. Perhaps more zinc was retained in the body of the lean rats as the study progressed. However, we observed a different trend among the fa/fa rats. Prior to the study, we assumed that the faZS group may show increased urine zinc excretion secondary to more zinc provided in the diet. However, the only fa/fa group that showed significant increases in urine zinc was the faPW rats. Here we demonstrated an *increase* in urinary zinc at the end of the study among the faPW rats compared to other treatment groups (Table 13 and Table 16). The pair-weighed fa/fa rats may have increased tissue catabolism associated with fasting between feeding periods (Heise et al., 1988). This increase in tissue catabolism may account for the higher urinary zinc levels. Our results imply that dietary zinc level is not associated with an increase in urine zinc excretion of the Zucker rat fed a zinc deficient, control or supplemented zinc diet. Zinc is also excreted in the feces and since we did not analyze the zinc content of the feces we can not report what effect our diet intervention had on this parameter.

Our results for urine zinc and zinc status of the fa/fa rats warrant further investigation. We demonstrated that zinc *excretion* is greater in the fa/fa rat compared to lean controls. However, the actual zinc *status* (femur and serum zinc concentrations) of the fa/fa rat is greater compared to lean rats (Table 10). The association between increases in urinary zinc losses in the presence of improved zinc status may be a reflection of increased intestinal absorption of zinc by the obese fa/fa rat. That is, the increased zinc excretion may be offset by increased absorption of zinc through the diet. Increased absorption of zinc has been demonstrated in ob/ob mice compared to lean mice by Kennedy and Failla (1987) but zinc absorption has not been investigated in the Zucker rat. However, according to our results for the small intestine (Table 7) the fa/fa rats have longer and heavier small intestines compared to lean rats, which may be an indication for increased absorption capabilities. Furthermore, the fa/fa rat may have decreased intestinal zinc excretion, a physiological response to conserve zinc in compensation for hyperzincuria.

Urinary glucose was also measured as an indication of insulin resistance. In addition to the usual absence of severe hyperglycemia, the Zucker rat also does not normally develop glucosuria (McCaleb and Sredy, 1992). Unexpectedly, after 9 weeks of dietary zinc intervention, urinary glucose showed significant genotype variability (Appendix E and Table 13). Despite similar *serum* glucose concentrations observed among the lean and fa/fa rats, the fa/fa rats excreted more glucose compared to their lean counterparts. On the contrary, McCaleb and Sredy (1992) showed that the fa/fa rat excreted 33 ± 7 mg/dl compared to 22 ± 2 mg/dl in the lean rats, a difference that did not reach statistical significance. Similarly, Koh et al., (1985) showed similar urine glucose

levels between 15-week old lean and fa/fa rats. Therefore, we saw similar urine glucose concentration for the fa/fa rat as reported in the literature but our study demonstrated lower levels of glucose excreted by the lean rats. In addition to our results, the fa/fa rats showed greater intra-animal variability (as shown by the standard error of the mean) implying that not all fa/fa rats had urine glucose levels as high as that indicated by the mean. The faPW rats excreted the highest amount of glucose at the end of the dietary intervention period (Table 13). Interestingly, this group also excreted the largest amount of zinc (Table 13). Perhaps the increases in these urinary parameters are consequences of impaired renal function in this particular fa/fa group. Dietary zinc supplementation did not alter glucose concentration of urine collected at the end of the study. On the contrary, Simon (1998) showed that 300 ppm zinc supplementation increased the urinary excretion of glucose in db/db mice. We used a lower level of dietary zinc a euglycemic rat model which may explain the differences observed between this study and Simon (1998).

Adipocyte Glut 4 Expression

The literature has shown that insulin stimulated glucose uptake into adipose cells is facilitated by Glut 4 and a reduction in Glut 4 may play a role in the development of insulin resistance (Pessin, 1992). The results of the current study show that the expression of total Glut 4 in adipocytes is lower in the fa/fa obese rat compared to lean controls. This observation is in agreement with the findings of Shmaya et al. (1997) and Kahn and Pederson (1993) for the fa/fa rat, with similar observations cited in patients with obesity and type 2 diabetes (Sinha et al., 1991; Rosenbaum et al., 1993; Garvey et al., 1991). Therefore, we can speculate that the insulin resistance observed in the fa/fa rats of our study may be associated with the reduced expression of adipocyte Glut 4.

We chose to study Glut 4 in isolated adipocytes given the hypothesis that zinc supplementation enhances body fat deposition. Glucose is a substrate for lipogenesis and enters the adipose tissue via the Glut 4. We speculated that zinc supplementation may increase the *expression* of Glut 4 in fat cells, thereby increasing the quantity of Glut 4 accessible for glucose uptake and hence, increasing the amount of glucose available for lipogenesis (body fat deposition). Interestingly, this study revealed a significant main effect of zinc opposite to what was expected. In the fa/fa genotype, zinc supplementation actually influenced a *reduction* in adipocyte Glut 4 expression. This effect of zinc was only observed in the fa/fa genotype and not in the lean zinc supplemented rats (Table 16). Surprisingly, the lower expression of Glut 4 in the faZS rats did not correspond to changes (increases) in serum glucose concentrations. Perhaps this was due to the fact that the fa/fa rat is a euglycemic model. Changes in serum glucose concentrations may have been manifested had we used a hyperglycemic animal model in our study, for example, the db/db mouse. The results for Glut 4 expression also lends us to believe that the Glut 4 present in the fa/fa rats (although less) was as effective in maintaining serum glucose concentrations within the same levels as the other dietary and genotype groups. Although zinc supplementation did not increase the *expression* of Glut 4 per se, it is possible that zinc supplementation improved the insulin signaling pathway. The binding of insulin to its receptor and the corresponding signal for Glut 4 translocation to the membrane may have improved with zinc supplementation. Most Glut 4 resides in intracellular membrane compartments until insulin stimulates the translocation of Glut 4 to the membrane surface where it contributes to cellular glucose uptake (Mueckler & Holman, 1995). Interestingly, King et al. (1992) demonstrated that the insulin resistance

of the fa/fa obese rat involves a failure of translocation of Glut 4 to the membrane. We did not study the translocation of Glut 4 in adipocytes of the zinc-supplemented rat, but this area warrants investigation.

We can conclude that if zinc supplementation does increase body fat deposition (as illustrated by Chen et al., 1998), we could not prove that it was via an increase in Glut 4 expression.

Although we chose to study the adipocytes, Glut 4 in the fa/fa rat is commonly studied in skeletal muscle tissue. Studies have shown both increases and decreases in the expression of Glut 4 in muscle tissues of obese/diabetic animal models (Kahn & Pederson, 1993; Kahn et al., 1991) and human subjects (Dohm et al., 1991; Pederson et al., 1990). At this stage of our research, we are not aware of what effects zinc supplementation had on the expression Glut 4 in the muscle but it is an area worth investigating.

The age of the rat may also be an influential factor on the expression of Glut 4 in adipocytes. Interestingly, Hainault et al. (1991) demonstrated a higher adipocyte Glut 4 expression in the obese rat compared to lean littermates. In comparison to our study design, the authors used fa/fa and lean pups (2-4 weeks of age) which is a significant age difference compared to our study (15 weeks of age). Nevertheless, Hainault et al. (1991) concluded that at a very young age, the expression Glut 4 in adipose is increased and plays a role in the progression of obesity. That is, the abundance of Glut 4 protein at the early stages of development entails a hyperresponsive glucose transport activity, providing large supplies of lipid synthesis precursors. Based on our research, we were able to elucidate the effects of dietary zinc intervention on Glut 4 of the adult rat.

Investigations on the effect of zinc on Glut 4 expression at an earlier stage warrants further research.

VI. Conclusions

Major Research Findings

- Weanling lean and obese (fa/fa) rats fed a 5 ppm zinc diet for 9 weeks develop marginal zinc deficiency as indicated by lower femur zinc concentrations. However, zinc supplementation (150 ppm zinc) did not elevate zinc concentration in serum, femur or liver.
- The obese (fa/fa) rats expressed less Glut 4 compared to lean littermates and diet (zinc supplementation, 150 ppm), combined with genetics (fa/fa) further reduced adipocyte Glut 4 expression in the Zucker rat, despite zinc supplementation not altering tissue zinc concentrations.
- Zinc supplementation (150 ppm) did not aggravate body or hepatic fat deposition, or adipocyte size in the Zucker rats.
- Zinc supplementation (150 ppm) did not attenuate hyperinsulinemia in the fa/fa rat.
- The fa/fa rats had higher femur and serum zinc concentrations, but lower hepatic zinc concentrations compared to lean rats. Zinc supplementation elevated serum zinc concentrations in lean Zucker rats but not serum zinc concentrations of fa/fa rats, implying altered zinc metabolism in the fa/fa genotype.

Implications for Future Research

Further research in the Zucker rat model is needed to reveal the effects of dietary zinc manipulation (deficiency and supplementation) on:

- The translocation of Glut 4 in adipocytes and/or muscle cells
- The expression of Glut 4 in muscle
- Glucose uptake studies in epididymal adipocytes
- The conversion rate of glucose to lipids in adipocytes.
- Liver lipid profiles and serum free fatty acids, triglycerides and cholesterol
- Glucose tolerance tests

VII. REFERENCES

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Appendix A

Main effects of genotype, zinc, and genotype x zinc interaction for total feed intake, body weight and body fat measurements of obese (fa/fa) and lean Zucker rats after 9 weeks of dietary treatment

MAIN EFFECTS[1]

	Genotype	Zinc	Genotype X Zinc
Total Feed Intake (g)	0.0001 *	0.7426	0.9861
Body Weight (g)	0.0001 *	0.2747	0.7021
Epididymal Fat Pad Weight (g)	0.0001 *	0.7906	0.1041
Perirenal Fat Pad Weight (g)	0.0001 *	0.6497	0.8252
Total Fat Pad Weight (g)	0.0001 *	0.8161	0.3573
Relative Epididymal Fat Weight (g/100 g body weight)	0.0001 *	0.6531	0.0251 *
Relative Perirenal Fat Weight (g/100 g body weight)	0.0001 *	0.5637	0.5595
Relative Total Fat Weight (g/100 g body weight)	0.0001 *	0.7460	0.0788
Adipocyte Diameter (*um*)	0.0001 *	0.2087	0.7775

[1] Values are Pr > F as assessed by ANOVA. * = significant main effect at $\alpha \leq 0.05$

Appendix B

Main effects of genotype, zinc, and genotype x zinc interaction for kidney, pancreas and spleen weight, and small intestine weight and length of obese (fa/fa) and lean Zucker rats after 9 weeks of dietary treatment

MAIN EFFECTS[1]

	Genotype	Zinc	Genotype X Zinc
Kidney Weight (g)	0.0001 *	0.5848	0.7878
Relative Kidney Weight (g/100 g body weight)	0.0001 *	0.5087	0.5389
Small Intestine Weight (g)	0.0001 *	0.3315	0.9910
Relative Small Intestine Weight (g/100 g body weight)	0.0001 *	0.9468	0.8155
Small Intestine Length (cm)	0.0005 *	0.3563	0.9344
Pancreas Weight (g)	0.0003 *	0.3265	0.9839
Relative Pancreas Weight (g/100 g body weight)	0.0001 *	0.8342	0.9010
Spleen Weight (g)	0.0001 *	0.3072	0.3072
Relative Spleen Weight (g/100 g body weight)	0.0001 *	0.4550	0.1081

[1] Values are Pr > F as assessed by ANOVA. * = significant main effect at $\alpha \leq 0.05$

Appendix C

Main effects of genotype, zinc, and genotype x zinc interaction for liver weights, liver lipid and liver copper of obese (fa/fa) and lean Zucker rats after 9 weeks of dietary treatment

MAIN EFFECTS[1]

	Genotype	Zinc	Genotype X Zinc
Liver Weight (g)	0.0001 *	0.8170	0.7718
Relative Liver Weight (g/100 g body weight)	0.0001 *	0.9706	0.5928
Liver Lipid Concentration (mg/g liver)	0.0001 *	0.8947	0.8380
Liver Lipid Content (g)	0.0001 *	0.8615	0.7986
Relative Liver Lipid Content (g/100 g liver)	0.0001 *	0.4150	0.5979
Liver Copper Concentration (*u*g/g dry weight)	0.0001 *	0.1103	0.6771
Total Liver Copper Content (mg/liver)	0.0001*	0.0861	0.9322
Relative Liver Copper Content (mg/100 mg liver)	0.7815	0.0552	0.8098

[1] Values are Pr > F as assessed by ANOVA. * = significant main effect at $\alpha \leq 0.05$

Appendix D

Main effects of genotype, zinc and genotype x zinc interaction for femur weight and length, zinc status and femur calcium and phosphorus of obese (fa/fa) and lean Zucker rats after 9 weeks of dietary treatment

MAIN EFFECTS[1]

	Genotype	Zinc	Genotype X Zinc
Femur Weight (g dry weight)	0.0456 *	0.6185	0.5340
Femur Length (cm)	0.0001 *	0.6732	0.6224
Femur Zinc Concentration (*u*g/g dry weight)	0.0001 *	0.0001 *	0.3835
Liver Zinc Concentration (*u*g/g dry weight)	0.0001 *	0.9344	0.7339
Total Liver Zinc Content (*u*g zinc/liver)	0.0001 *	0.4198	0.9201
Relative Liver Zinc content (mg/100 mg liver)	0.0001 *	0.9579	0.4792
Serum Zinc Concentration (*u*g/ml)	0.0001 *	0.0366 *	0.1553
Femur Calcium (mg/g dry weight)	0.0001*	0.0001*	0.0001*
Femur Phosphorus (mg/g dry weight)	0.0001*	0.0001*	0.0001*

[1] Values are Pr > F as assessed by ANOVA. * = significant main effect at $\alpha \leq 0.05$

Appendix E

Main effects of genotype, zinc, and genotype x zinc interaction for serum and urine indices of diabetes and adipocyte total Glut 4 of obese (fa/fa) and lean Zucker rats after 9 weeks of dietary treatment.

MAIN EFFECTS[1]

	Genotype	Zinc	Genotype X Zinc
Serum Insulin (ng/ml)	0.0001 *	0.7439	0.6961
Serum Glucose (mmol/L)	0.1453	0.1815	0.4360
Urine Volume (ml/12 hours)	0.1971	0.4476	0.7671
Urine Creatinine (mg/12 hours)	0.0038 *	0.1505	0.0026 *
Urine Zinc (mg zinc/mg creatinine)	0.0001 *	0.2613	0.2107
Urine Glucose (mg glucose/mg creatinine)	0.0242*	0.6377	0.6339
Glut 4 (arbitrary units)	0.0025 *	0.0032 *	0.0697

[1] Values are Pr > F as assessed by ANOVA. * = significant main effect at $\alpha \leq 0.05$

Appendix F

Effects of dietary zinc on body weight of obese (fa/fa) and lean Zucker rats at weeks 0, 3, 6 and 9 of dietary treatment[1]

	Dietary Treatment Groups[2]							
	fa/fa				lean			
	faZD	faZC	faPW	faZS	lnZD	lnZC	lnPW	lnZS
Body Weight Week 0 (g)	192[d] ±11	198[d] ±11	193[d] ±12	196[d] ±10	121[d] ±5	122[d] ±6	121[d] ±4	126[d] ±4
Body Weight Week 3 (g)	396[c] ±13	407[c] ±11	406[c] ±15	408[c] ±12	236[c] ±7	255[c] ±5	253[c] ±6	264[c] ±6
Body Weight Week 6 (g)	535[b] ±18	555[b] ±15	529[b] ±16	550[b] ±16	305[b] ±8	316[b] ±9	310[b] ±8	335[b] ±10
Body Weight Week 9 (g)	608[a] ±22	620[a] ±18	599[a] ±15	619[a] ±19	350[a] ±11	368[a] ±13	359[a] ±11	387[a] ±10

[1] Values represent the mean ± SEM for n=7 rats. Different superscript letters indicate significant differences between **means of different weeks within the same treatment group** as determined by Duncan's multiple range test.

[2] fa/fa=obese Zucker rat, lean=lean Zucker rat, faZD=fa/fa zinc deficient diet, faZC=fa/fa zinc control diet, faPW=fa/fa pair-weighed, faZS=fa/fa zinc supplemented diet, lnZD=lean zinc deficient diet, lnZC=lean zinc control diet, lnPW=lean pair-weighed and lnZS=lean zinc supplemented diet.

Appendix G

Main effects of time, dietary treatment, and time x dietary treatment interaction for urine volume, creatinine, zinc and glucose (**within treatment groups over time**) of obese (fa/fa) and lean Zucker rats after 9 weeks of dietary treatment.

MAIN EFFECTS[1]

	Time	Dietary Treatment	Time x Dietary Treatment
Urine Volume (ml)	0.0005 *	0.0001 *	0.9916
Urine Creatinine (mg/12 hours)	0.0001 *	0.0003 *	0.0001 *
Urine Zinc (mg zinc/mg creatinine)	0.0308 *	0.0001 *	0.0309 *
Urine Glucose (mg glcuose/mg creatinine)	0.4748	0.2228	0.0.5299

[1] Values are Pr > F as assessed by ANOVA. * = significant main effect at $\alpha \leq 0.05$