THE EFFECTS OF MARGINAL ZINC DEFICIENCY AND ZINC SUPPLEMENTATION ON DIABETES AND THE IMMUNE SYSTEM IN ZUCKER DIABETIC FATTY RATS

by

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ABSTRACT

Many of the symptoms of zinc deficiency and diabetes are the same, and are related to increased inflammation. Zinc supplementation may improve immunity and glycemic control, and reduce inflammation. The purpose of this study was to determine if marginal zinc deficiency (MZD) worsens and zinc supplementation (ZS) improves diabetes and immune parameters in Zucker diabetic fatty (ZDF) rats. Male ZDF rats were fed MZD (4 mg Zn/kg diet), zinc control ([ZC], 30 mg Zn/kg diet), or ZS (300 mg Zn/kg diet) diet, and lean ZDF rats fed ZC diet for 8 weeks. Parameters of diabetes, inflammation, and immune cell proportions and function were assessed. Results showed that MZD may exacerbate diabetes but had little effect on the immune parameters, while ZS had little effect on diabetes but may worsen immune function. Overall, it is the balance between zinc deficiency and toxicity that is necessary for optimal health improvements.

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AA	Aminoantipyrine
Ab	Antibody
AAS	Atomic absorption spectroscopy
ACOD	Acyl-CoA-oxidase
Acyl-CoA	Acyl-coenzyme A
Acyl CS	Acyl-CoA synthetase
AIN	American Institute of Nutrition
AMP	Andeosine-5'-monophosphate
ANOVA	Analysis of variance
APS	Ammonium persulfate
ATP	Adenosine-5'-triphosphate
AUC	Area-under-the-curve
BCA	Bicinchoninic acid
BMI	Body mass index
BPB	Bromophenol blue
BSA	Bovine serum albumin
BW	Body weight
CO_2	Carbon dioxide
CRP	C-reactive protein
CV	Coefficient of variance
ddH_2O	Double distilled water
DEXA	Dual-energy X-ray absorptiometry

DNA	Deoxyribonucleic acid
Enoyl-CoA	2,3-enoyl-coenzyme-A
FPG	Fasting plasma glucose
FPI	Fasting plasma insulin
FFA	Free fatty acid
GLUT	Glucose transporter
GOD-POD	Glucose oxidase/peroxidase
GPR-39	G-protein coupled receptor-39
H_2O	Water
H_2O_2	Hydrogen peroxide
Hb_{A1C}	Glycated haemoglobin
HCl	Hydrogen chloride
HDL-C	High-density lipoprotein cholesterol
HEPES	4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid
HOMA-BCF	Homeostasis model assessment of insulin resistance
HOMA-IR	Homeostasis model assessment of beta-cell function
HRP	Horseradish peroxidase
ICP-OES	Inductively coupled plasma-optical emission spectrometry
IL	Interleukin
IFN-γ	Interferon-γ
ISI	Insulin sensitivity index
JAK	Janus kinase
LDL-C	Low-density lipoprotein cholesterol

MCP-1	Monocyte chemoattractant protein-1
β-ME	2-mercaptoethanol
MSD	Meso-Scale Discovery
MZD	Marginal zinc deficiency
NaCl	Sodium chloride
ND	Not detectable
NFκB	Nuclear factor kappa-light-chain-enhancer of activated B cells
O_2	Oxygen
OGTT	Oral glucose tolerance test
O/N	Overnight
PBS	Phosphate-buffered saline
POD	Peroxidase
PVDF	Polyvinylidene fluoride
PW	Pair-weighed
QMR	Quantitative magnetic resonance
RNA	Ribonucleic acid
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SDT	Spontaneously diabetic torii
SOCS-3	Suppressor of cytokine signaling-3
STAT	Signal transducer and activator of transcription
TBHB	2,4,6-tribromo-3-hydroxy-benzoic acid
TBST	Tris-buffered saline with Tween-20

TEMED	N,N,N’,N’-Tetramethylethlylenediamine
TG	Triglycerides
TLR	Toll-like receptor
TNF-α	Tumor necrosis factor-α
ZC	Zinc control
ZD	Zinc deficient
ZDF	Zucker diabetic fatty
ZIP	Zrt and irt-like protein or solute-linked carrier 39
ZnT	Solute-linked carrier 30
ZR	Zinc restricted
ZS	Zinc supplemented

I. LITERATURE REVIEW

The aim of this study was to assess the effect of zinc status on parameters for both diabetes and immune system function. Studies have been performed assessing the effect of zinc status in diabetes, or zinc and the immune system, but no studies to our knowledge have looked at the effect of zinc on a combination of both in a diabetes model. The following literature review will outline the information known about zinc's relation to diabetes, and zinc and the immune system, as well as assess the research gaps.

Zinc

Zinc is an essential trace mineral required for the functioning of over 300 enzymes involved in the structural integrity of proteins and organelles, metallothionein, immune function, insulin metabolism, and stabilization of RNA and DNA (Islam & Loots du, 2007; Jansen, Karges, & Rink, 2009; John *et al.*, 2010; Wiernsperger & Rapin, 2010). Zinc is consumed through the diet as it is found in most foods, ingested, released in the stomach, and absorbed in the small intestine through carrier mediated transport and passive diffusion (Maret & Sandstead, 2006; Sandstrom, 1997).

Zinc deficiency can be caused by inadequate intake or by malabsorption syndromes such as acrodermatitis enteropathica or Crohn's disease (Maret & Sandstead, 2006; Sandstrom, 1997). It is estimated that about 20% of the world's population is at risk for inadequate zinc intake and that zinc deficiency is a major contributor to the worldwide burden of disease (Ezzati *et al.*, 2002; Wuehler, Peerson, & Brown, 2005). It is estimated that mild zinc deficiency is highly prevalent in both developed and developing countries (Wuehler, Peerson, & Brown, 2005). Symptoms of zinc deficiency

include growth retardation, immune dysfunction, weight loss, skin lesions, thymic atrophy, neurological disorders and infertility (Plum, Rink, & Haase, 2010). Zinc deficiency usually occurs from inadequate intake, while zinc toxicity results from pharmacological zinc supplementation (Maret & Sandstead, 2006). Excessive zinc intake results in impaired copper and iron absorption, altered lymphocyte function, nausea, vomiting, and lethargy (Plum, Rink, & Haase, 2010; Sandstrom, 2001).

Zinc homeostasis is maintained through changes in absorption and excretion in the intestine (Sandstrom, 1997). Zinc absorption depends on its bioavailability and the zinc content of the food or solution; at low zinc content greater than 50% of fractional zinc can be absorbed but higher total zinc absorption occurs with high zinc content (Sandstrom, 1997). The rate of zinc loss through the urine, skin and intestine is constant and will only change during low or excessive zinc intake (Sandstrom, 1997). Plasma concentrations of zinc are tightly regulated by zinc transporters and zinc binding proteins to keep normal plasma levels around 12 to 16 μM (Jansen, Karges, & Rink, 2009). Plasma zinc is usually found bound to albumin, α-microglobulin, and transferrin (Plum, Rink, & Haase, 2010; Wiernsperger & Rapin, 2010). The human body contains 2 to 3 grams of zinc which is distributed throughout the body, with approximately 95% of zinc being intracellular (Chimienti, Seve, Richard, Mathieu, & Favier, 2001; King, 2011). Most zinc in the body is bound to proteins, with only small pools of free zinc within cells and the plasma (King, 2011). Bone acts as a storage reservoir for zinc and femur zinc concentrations reflect zinc status (Zhou, Canar, & Erdman, 1993).

There are two main classes of zinc transporters in cells: ZIP (Zrt- and Irt- like proteins or solute-linked carrier; 39) which are responsible for zinc influx into cells, and

ZnT (solute-linked carrier; 30) which are responsible for zinc efflux from the cytosol into intracellular organelles and from the cytosol into extracellular space (Lemaire *et al.*, 2009). There are 14 members of the ZIP family and 10 members of the ZnT family (Egefjord, Petersen, & Rungby, 2010; Plum, Rink, & Haase, 2010).

Metallothioneins are responsible for a significant amount of zinc binding as they form complexes with up to 20% of available intracellular zinc (Plum, Rink, & Haase, 2010). There are 4 major isoforms of metallothionein: metallothionein-1 and metallothionein-2 are located throughout the body, metallothionein-3 is primarily found in the brain, and metallothionein-4 is found in stratified tissues (King, 2011). One metallothionein molecule can bind up to 7 zinc ions, therefore, metallothionein acts as a cellular zinc buffer (Plum, Rink, & Haase, 2010). Overall, zinc levels are tightly regulated within the body and any disruption to this balance negatively impacts the body's functioning.

Diabetes

Diabetes, rapidly increasing in prevalence, is the most common endocrine disease worldwide with over 177 million people diagnosed (Islam & Loots du, 2007). Diabetes is a disease that is characterized by chronically high fasting (≥7 mmol/L) or postprandial blood glucose levels (≥11.1 mmol/L), altered lipid metabolism and oxidative stress (Alberti & Zimmet, 1998; Islam & Loots du, 2007; Kanoni *et al.*, 2011). Type 1 diabetes accounts for 10% of the diabetic population and is due to a lack of insulin production, while type 2 diabetes accounts for 90% of the diabetic population and is due to insulin resistance (Chausmer, 1998).

The main hormones involved in glycemic regulation are insulin and glucagon. Insulin is released from pancreatic β-cells and travels in the bloodstream to tissues where it stimulates glucose uptake, glycogenesis, and lipogenesis (Gual, Le Marchand-Brustel, & Tanti, 2005). Insulin inhibits gluconeogenesis and glycogenolysis in the liver to prevent glucose release into the blood in the postprandial state (Saltiel & Kahn, 2001). Elevated levels of circulating insulin are associated with type 2 diabetes (Gual, Le Marchand-Brustel, & Tanti, 2005). Hepatic insulin resistance is primarily caused by an excessive accumulation of fat in the liver (Roden, 2006). Furthermore, hepatic steatosis is common in type 2 diabetes and may precede or exacerbate hepatic insulin resistance (Roden, 2006; Schmid *et al.*, 2011). Excess liver fat leads to an activation of inflammatory pathways and cirrhosis of the liver (Roden, 2006). As type 2 diabetes progresses, a reduction in islet cell mass is common leading to a decline in insulin production (Jansen, Karges, & Rink, 2009).

Zinc and Diabetes

Zinc plays an important role in metabolic disorders such as insulin resistance, metabolic syndrome, and diabetes since it is required for insulin maturation, crystallization and secretion from pancreatic β-cells (Li, 2013; Wiernsperger & Rapin, 2010). Zinc is involved in diabetes and glycemic control since it is co-secreted with insulin and acts on α-cells to inhibit glucagon secretion (Ishihara, Maechler, Gjinovci, Herrera, & Wollheim, 2003). Consequently, hyperinsulinemia may contribute to zinc deficiency in people with type 2 diabetes.

Many of the signs of zinc deficiency and diabetes are similar; they both present with impaired insulin signalling, oxidative stress, impaired wound healing and immune dysfunction, altered lipid metabolism, and infertility (Alberti & Zimmet, 1998; Forbes & Cooper, 2013;Islam & Loots du, 2007; Kanoni *et al.*, 2011; Plum, Rink, & Haase, 2010). There is a high prevalence of zinc deficiency as well as type 2 diabetes in Africa, suggesting an important link between the two disorders (Prasad, 1996; Umeta, West, Verhoef, Haidar, & Hautvast, 2003). Hyperglycemia reduces the activity and binding of zinc to the metal responsive element found on the promoter region of certain genes which in turn reduces the transcription of several genes related to zinc homeostasis and protection against oxidative stress (Yi *et al.*, 2012).

The main characteristic of both type 1 and type 2 diabetes is hyperglycemia which leads to polyuria and consequently, hyperzincuria and hypozincemia (Chausmer, 1998; Jansen, Karges, & Rink, 2009). Urinary zinc levels can be used as an indicator of the extent of diabetes since hyperzincuria occurs and becomes exacerbated with progressing diabetes (Chausmer, 1998; Jansen, Karges, & Rink, 2009). A review by Chausmer (1998) illustrated that reduced serum zinc concentrations are common in individuals with diabetes. As a result, serum zinc levels have been found to be lower in patients with diabetes than those without diabetes (Gunasekara, Hettiarachchi, Liyanage, & Lekamwasam, 2011). Therefore, zinc deficiency (classified as serum zinc levels below 10.7 μmol/L) is common in people with diabetes and contributes to associated complications, such as nephropathy, as well as limiting glucose-stimulated insulin secretion (Chausmer, 1998; Seet *et al.*, 2011).

Zinc assists in the secretion of leptin, a hormone produced by adipose tissue and involved with control of appetite (gives the feeling of satiety). Consequently, zinc deficiency contributes to a decrease in leptin production and secretion from adipocytes (Ott & Shay, 2001; Smidt *et al.*, 2007). Basuki *et al.* (2007) have shown that zinc complexes (bis[1-oxy-2-pryridine-thiolato]Zn [II]) induce Akt/PKB phosphorylation in 3T3-L1 adipocytes and act as an insulin mimetic. Tang and Shay (2001) found that zinc increases tyrosine phosphorylation of the insulin receptor and activates Akt (which is required for the insulin receptor to function) enhancing insulin signalling. Tang and Shay (2001) treated 3T3-L1 adipocytes with zinc, insulin, and a combination of both and measured glucose transport. They discovered that zinc alone was able to increase glucose uptake in adipocytes, but zinc plus insulin had the greatest effect on glucose uptake (Tang & Shay, 2001). Therefore, zinc complexes mimic insulin's action through the insulin signalling pathway and translocation of GLUT 4 from the cytosol to the plasma membrane to stimulate uptake of glucose (Basuki, Hiromura, & Sakurai, 2007; Tang & Shay, 2001; Thirumoorthy *et al.*, 2011).

G protein-coupled receptor-39 (GPR-39) is a zinc sensing receptor and a member of the ghrelin family that is activated by zinc, thereby inducing signal transduction pathways (Egerod *et al.*, 2011; Mlyniec, Budziszewska, Reczynski, Sowa-Kucma, & Nowak, 2013). GPR-39 is found in peripheral tissues with endocrine and metabolic functions and it is involved in the control of appetite, the functioning of the gastrointestinal tract, energy production and metabolism (Egerod *et al.*, 2011). GPR-39 deficiency has been associated with islet cell dysfunction, impaired insulin secretion in

response to glucose, and glucose intolerance (Holst *et al.*, 2009). Thus, zinc status may alter GPR-39 activation, islet cell function, and glucose tolerance.

Zinc efflux from cells is controlled by ZnT transporters which are responsible for transporting zinc from the cytosol into intracellular organelles and from the cytosol to extracellular space (Lemaire *et al.*, 2009). Hyperglycemia reduces mRNA levels of the zinc transporter, ZnT2, in rat hearts (Yi *et al.*, 2012). ZnT8 is a member of the zinc solute transporter family and found almost exclusively in pancreatic islet cells (Lemaire *et al.*, 2009). Lemaire *et al.* (2009) analyzed islet cells from ZnT8 null mice to determine the role of the transporter in insulin response. The results indicated that ZnT8 null nice exhibit a loss of zinc release from secretory granules, a replacement of dense-core insulin granules by larger, pale, less dense granules, and a loss of glycemic control when fed a high fat diet (Lemaire *et al.*, 2009). Although, in zinc deficiency, the pancreas is able to maintain zinc levels in islets cells, even though total pancreatic zinc content may be reduced (Sondergaard, Stoltenberg, Doering, Flyvbjerg, & Rungby, 2006).

RNA levels of zinc transporters (ZnT and ZIP) vary in lean versus obese humans (Smidt *et al.*, 2007). Subcutaneous adipose tissue contains higher levels of most zinc transporters than visceral fat, with higher levels in lean humans compared to obese (Smidt *et al.*, 2007). ZnT2, ZnT3, ZnT6, and ZnT8 were expressed at higher levels in subcutaneous fat than visceral fat, and in fat tissue from lean versus obese individuals (Smidt *et al.*, 2007). The different levels of zinc transporter expression between lean and obese individuals suggest that obesity can influence zinc transport and vice versa (Smidt *et al.*, 2007). Thus, obesity reduces adipocyte zinc transporter expression and potentially, zinc content (Tallman & Taylor, 2003). Also, the varying levels of zinc transporters

between subcutaneous and visceral fat suggests that the metabolic activity of adipose tissue may be influenced by zinc (Smidt *et al.*, 2007).

Zinc has the ability to stimulate lipogenesis in adipocytes, in the absence of insulin (Coulston & Dandona, 1980). Zinc also helps to regulate the metabolism of adipose tissue by assisting in leptin secretion, the release of free fatty acids, and glucose uptake (Coulston & Dandona, 1980; Ott & Shay, 2001; Tang & Shay, 2001). Zinc may play an important role in diabetes by regulating circulating free fatty acids levels, which are elevated in obesity and type 2 diabetes (Jialal, Huet, Kaur, Chien, & Devaraj, 2012). As described above, zinc transporter expression is altered in obesity and this may impair zinc metabolism and homeostasis as well as adipocyte function.

Zinc may have many protective effects against diabetes and its associated co-morbidities in humans. In a prospective dietary intake study done by Sun *et al.* (2009) using women in the United States, the highest quintiles for both total and dietary zinc intake were significantly associated with a 20% lower risk of developing type 2 diabetes. Dyslipidemia is common among people with diabetes and contributes to cardiovascular complications (Talayero & Sacks, 2011). Gunasekara *et al.* (2011) found that zinc supplementation (22 mg/day) for 4 months significantly reduced blood glucose, serum lipids, total and low-density lipoprotein (LDL) cholesterol, and glycated haemoglobin (Hb_{A1C}) levels in humans with type 2 diabetes. Therefore, zinc supplementation may reduce the risk of developing co-morbidities in people with diabetes through improved blood lipid profile and glycemic control. In an *ex vivo* study, Brender *et al.* (2010) discovered that zinc (even in millimolar concentrations) significantly inhibits the aggregation of human islet amyloid polypeptide, a highly toxic, amyloidogenic protein

found in the islet cells of patients with type 2 diabetes. Based on the *ex vivo* study, it is predicted that reducing the aggregation of this protein would help to prevent the loss of β-cell functioning and improve insulin secretion (Brender *et al.,* 2010). Therefore, zinc may have powerful anti-diabetes properties even at low concentrations within cells.

Zinc Deficiency and Supplementation in Rodent Models of Type 2 Diabetes

Dietary zinc deficiency in animal models has been able to demonstrate the importance of zinc in relation to diabetes. Firstly, obese diabetic animals have altered zinc status. Simon and Taylor (2001) found that pancreatic zinc concentrations were lower in obese, diabetic (*db/db*) mice and *db/db* mice exhibit elevated serum insulin and urinary glucose excretion compared to their lean controls. Although, zinc deficiency (3 mg Zn/kg diet) did not alter pancreatic or femur zinc concentrations compared to zinc control *db/db* mice (Simon & Taylor, 2001).

A review by Taylor (2005) found that zinc deficiency may exacerbate diabetes, while zinc supplementation tends to attenuate diabetes in rodents. Tallman and Taylor (2003) found that zinc deficiency of 3 mg Zn/kg diet in weanling C57BL/6J mice reduced the amount of fatty acids per gram of adipose tissue and there was a negative correlation between adipose zinc concentrations and serum leptin. The authors suggest that these findings demonstrate an interrelationship among obesity, leptin and zinc metabolism (Tallman & Taylor, 2003).

Although, the majority of studies assessing dietary zinc intake and diabetes focus on glycemic control and pancreatic function. Soondergaard *et al.* (2006) found that female Wistar rats fed a zinc deficient (<10 mg Zn/kg diet) diet for 4 weeks had

marginally impaired glucose metabolism and elevated blood glucose levels but there was no difference in serum insulin, insulin resistance, or beta-cell function. Meanwhile, Huber and Gershoff (1973) showed that dietary zinc did not affect pancreatic insulin content but zinc deficiency (1 mg Zn/kg diet) reduced serum insulin and total serum insulin-like activity. In addition, zinc content within pancreatic islet cells was unaffected by zinc deficiency, while acinar cells and total pancreatic zinc content was reduced (Sondergaard, Stoltenberg, Doering, Flyvbjerg, & Rungby, 2006). This data suggests that the pancreas can compensate for reduced zinc availability to maintain beta-cell function, even though glucose metabolism is negatively impacted by marginal zinc deficiency. Further, Nicolson *et al.* (2009) discovered that deletion of the zinc transporter, ZnT8 (which is primarily found in the pancreas), in mice results in glucose intolerance, altered insulin secretion, and weight gain. Therefore, zinc is crucial for proper pancreatic function and to maintain blood glucose levels. In addition, the extent to which the consequences of zinc deficiency are displayed depend on the level of dietary zinc intake and zinc status within the body.

Zinc supplementation has been demonstrated to have many protective effects in rodent models of diabetes. Simon and Taylor (2001) found a negative correlation between serum glucose and dietary zinc intake. They showed that zinc supplementation (300 mg Zn/kg diet) in *db/db* mice can restore pancreatic zinc concentrations to those of lean mice, and elevate femur zinc concentrations compared to both *db/db* and lean mice fed zinc adequate control diet (Simon & Taylor, 2001). Furthermore, Simon and Taylor (2001) discovered that dietary zinc supplementation can attenuate fasting hyperglycemia, hyperinsulinemia, and reduce body weight compared to a zinc control diet in *db/db* mice.

In C57BL/6J mice, serum leptin negatively correlated with adipose tissue zinc concentrations; adipose tissue zinc concentrations were lower in high-fat fed mice than low-fat fed mice but was not affected by dietary zinc intake (Tallman & Taylor, 2003). A review by Islam and Loots du (2007) found that zinc supplementation improved hyperlipidemia, hyperinsulinemia, and hyperglycemia in diabetic rodents. In addition, zinc supplementation prior to streptozotocin injection was able to prevent the development of diabetes in mice (Islam & Loots du, 2007). The review illustrates that oral zinc supplementation increases the absorption and efficacy of zinc better than intra-peritoneally injected zinc (Islam & Loots du, 2007). Although, the appropriate level of zinc supplementation is still yet to be determined. In *ob/ob* mice, high levels of dietary zinc supplementation (1000 mg Zn/kg diet) for 4 weeks lowered fasting blood glucose and insulin concentrations, and but did not improve glucose tolerance compared to controls (Begin-Heick, Dalpe-Scott, Rowe, & Heick, 1985). Therefore, high dose zinc supplementation may not improve glucose uptake in peripheral tissues. In addition, high dose zinc supplementation is associated with copper deficiency because zinc and copper compete for absorption (Plum, Rink, & Haase, 2010). Therefore, the dose and method of administration of zinc supplementation can affect the level of benefit and more research is needed to establish the appropriate level of supplementation.

Some relevant animal studies involving zinc and diabetes are summarized in Table 1. Zinc supplementation (300 mg Zn/kg diet) was found to lower blood glucose after 6 weeks in *db/db* mice (Simon & Taylor, 2001) and reduce lipid peroxidation (1000 mg Zn/kg diet) after 8 weeks in New Zealand white rabbits without zinc deficiency (Jenner *et al.,* 2007). Meanwhile, other studies have found mixed results for the effect of

zinc on blood glucose: zinc deficiency may lower (5 mg Zn/kg diet, Petroulakis, 2000), have no effect (10 mg Zn/kg diet, Padmavathi *et al.*, 2009), or elevate (<10 mg Zn/kg diet, Sondergaard, Stoltenberg, Doering, Flyvbjerg, & Rungby, 2006) serum glucose in rats. There is also controversial evidence for zinc's role in insulin secretion. Huber and Gershoff (1973) found that zinc deficiency (1 mg Zn/kg diet) reduced serum insulin and improved adipose tissue response to insulin, meanwhile, Soondergaard *et al.* (2006) found that zinc did not affect circulating insulin levels, and Padmavathi *et al.*, (2009) found that maternal zinc restriction elevated fasting plasma insulin levels in offspring of Wistar rats. Zinc deficiency has also been shown to reduce the expression of the *ob* gene and leptin secretion (Ott & Shay, 2001). Most of these studies documented zinc status by reporting femur zinc concentrations (Petroulakis, 2000; Tallman & Taylor, 2003; Zhou, Canar, & Erdman, 1993). Therefore, it has been demonstrated that zinc is involved in multiple aspects of diabetes but inconsistent results among studies indicates that more research is needed to determine the dose and mechanisms of action.

Table 1. Summary of Animal Studies Relating to Zinc and Diabetes Parameters

Reference	Model	Diet/Groups	Length of Study	Parameters		
				Glucose	Insulin	Other
Huber & Gershoff, 1973	Male weanling CD rats (4 weeks old)	Zinc deficient (1 mg Zn/kg diet), zinc adequate (20 mg Zn/kg diet), zinc supplemented (1200 mg Zn/kg diet), pair fed	3 weeks		↓ serum insulin and total serum insulin-like activity in zinc deficiency, adipose tissue response to insulin ↑ in ZD rats; ZS ↑ the release of total insulin-like activity	
Petroulakis, 2000	Male lean and *fa/fa* Zucker rats (6 weeks old)	Zinc deficient (5 mg Zn/kg diet), adequate zinc (30 mg Zn/kg diet), zinc supplemented (150 mg Zn/kg diet)	9 weeks	↓ serum glucose in ZD *fa/fa* vs. ZS lean	No effects of zinc on serum insulin in both *fa/fa* and lean rats	ZD ↓ femur zinc concentrations in both lean and *fa/fa* rats; ZS did not affect femur zinc in both lean and *fa/fa* rats; *fa/fa* rats had 18% higher femur zinc concentrations than lean rats

Ott & Shay, 2001	Male Sprague-Dawley rats (6 weeks old)	Zinc deficient (1 mg Zn/kg diet), zinc adequate (20 mg Zn/kg diet), pair-fed	21 days			ZD ↓ expression of the *ob* gene and leptin secretion
Simon & Taylor, 2001	Female lean and *db/db* mice (4 weeks old)	Zinc deficient (3 mg Zn/kg diet), zinc adequate (30 mg Zn/kg diet), zinc supplemented (300 mg Zn/kg diet)	6 weeks	↓ fasting serum glucose in ZS vs. ZD in *db/db* mice, femur zinc negatively correlated with serum glucose in lean mice	ZS and ZD ↓ circulating insulin concentrations vs. ZC in *db/db* mice	
Tallman & Taylor, 2003	Male C57BL/6J mice (4 weeks old)	Low or high fat diet containing: zinc deficient (3 mg/kg diet), zinc control (30 mg/kg diet), or zinc supplemented (150 mg/kg diet)	16 weeks	No effect	No effect	Femur zinc concentrations ↑ in ZS group in both low and high fat fed mice; high fat groups had ↓ adipose zinc levels vs. low fat groups

Sondergaard, Stoltenberg, Doering, Flyvbjerg, & Rungby, 2006	Female Wistar rats (8 weeks old)	Zinc deficient (<10 mg Zn/kg diet), zinc supplemented (70 mg Zn/kg diet), pair fed	4 weeks	ZD ↑ blood glucose concentrations	No effect of ZD on insulin levels	ZD did not affect levels of zinc in islet cells but ↓ serum zinc
Jenner *et al.*, 2007	New Zealand white rabbits (~12 weeks old)	Normal diet (60 mg Zn/kg diet), high cholesterol (1% wt/wt cholesterol 50 mg Zn/kg diet), high cholesterol and zinc supplemented (1% wt/wt cholesterol, 1 g Zn/kg diet)	8 weeks			ZS ↓ accumulation of total cholesterol levels in aorta and cholesterol oxidation products
Padmavathi *et al.*, 2009	Female Wistar/NIN rats (70 days old)	Zinc restricted (ZR, 10 mg Zn/kg diet), zinc control (35 mg Zn/kg diet). ZR or ZC 2 weeks prior and during pregnancy; then ZR→ZC or ZR stayed ZR until weaning; then ZR→ ZC or ZR stayed ZR	2 weeks before pregnancy to180 day old offspring	No effect of maternal diet on fasting plasma glucose in offspring	Maternal ZR ↑ fasting plasma insulin levels in offspring and ↓ glucose-stimulated insulin secretion during glucose tolerance test on 90 and 180 day old female offspring	Maternal ZR ↑ body fat, ↓ lean mass of offspring

The Immune System

The immune system comprises a series of responses performed by specialized cells that are responsible for defending against infection and foreign invading microbes (Delves & Roitt, 2000). The immune system is composed of two main pathways: the innate and adaptive immune system. The innate immune system is the body's first line of defence against pathogens (Delves & Roitt, 2000; John *et al.*, 2010). It involves natural killer cells and macrophages which are immune cells that recognize pathogens through pattern-recognition receptors expressed on cell surface membranes and activate an inflammatory response and the production of cytokines (Calle & Fernandez, 2012; John *et al.*, 2010). The adaptive immune response involves both T- and B-lymphocytes; B-lymphocytes induce humoral immunity and produce antibodies, while T-lymphocytes induce cell-mediated immunity using helper ($CD4^+$) and cytotoxic ($CD8^+$) T-lymphocytes (John *et al.*, 2010). T- and B-lymphocytes are produced in the bone marrow; B-lymphocytes mature in the bone marrow and are released into circulation, while T-lymphocytes travel to the thymus for maturation and then enter the blood and secondary lymphoid tissue such as the spleen, where they respond to specific antigens upon stimulation (Delves & Roitt, 2000). Immune cells can be distinguished by cell surface phenotype markers: T-lymphocytes ($CD3^+$, $CD4^+$, $CD8^+$), B-lymphocytes ($CD45^+$), monocytes ($CD3^-CD4^+$), and natural killer cells ($CD3^-CD8^+$) (BD Biosciences, 2012; Hosseinzadeh & Goldschneider, 1993; Ruth, Proctor, & Field, 2009). In rats, recently released T-lymphocytes from the thymus are identified by CD90 which lasts for about 3 to 7 days on the cell surface (Hosseinzadeh & Goldschneider, 1993).

Zinc and the Immune System

Zinc is required for the physiological functioning of both the innate and adaptive immune systems, and especially for the development and maturation of T-lymphocytes (Jansen, Karges, & Rink, 2009). The first consequence of zinc deficiency is impaired immune function; levels of circulating cytokines (produced from lymphocytes, granulocytes, and platelets) fluctuate in response to altered zinc status before a change in plasma zinc concentration can be detected (Foster & Samman, 2012; Prasad, 2008). It has been proposed that zinc deficiency adversely affects lymphocyte proliferation and functioning through elevated glucocorticoids causing thymic atrophy, accelerated apoptosis and reduced lymphopoiesis (John *et al.*, 2010; Taylor & Giesbrecht, 2000). Zinc inhibits apoptosis by preventing the activation of caspase-3 which is a potent protease activator of programmed cell death (Chimienti, Seve, Richard, Mathieu, & Favier, 2001). Although, Hosea (2006) demonstrated contradictory results where lymphocytes from zinc deficient rats did not show increased levels of apoptosis. Hosea (2006) proposed that zinc deficiency, apart from elevated glucocorticoids, alters the balance of Th1 to Th2 cytokines leading to a pro-inflammatory state. Meanwhile, Sazawal *et al.* (1997) demonstrated that oral zinc supplementation for 120 days in children increased the number of $CD3^+$ and $CD4^+$ cells, and the CD4:CD8 ratio suggesting improved cell-mediated immunity.

Zinc affects the levels of cytokines though altered gene expression in T-lymphocytes by altering the balance of Th1 and Th2 populations (John *et al.*, 2010). Th1 cells produce interferon-gamma (IFN)-γ, interleukin (IL)-2, and TNF-α which enhance cell-mediated immunity, whereas Th2 cells produce IL-4, IL-6, and IL-10 which enhance

humoral immunity (DiPiro, 1997; John *et al.*, 2010). Bao *et al.* (2003) reported that zinc deficiency alters cellular immune response leading to increased microbial infections, thymus atrophy, decreased natural killer and thymic hormone activity, as well as altered cytokine production. Cytokines such as IFN-γ and IL-1β contribute to a down-regulation of zinc transporter expression and insulin gene expression in islet cells (Egefjord *et al.*, 2009). Of the zinc transporters, the ZnT8 transporter, which is almost exclusively found in pancreatic islet cells, was down-regulated approximately 12-fold by cytokines (Egefjord *et al.*, 2009). Down-regulation of zinc transporters by cytokines limits the functional capacity of the cell and the availability of zinc for other processes such as thymulin production. Thymulin is a hormone secreted by the thymus that requires zinc as a cofactor and is essential for T-lymphocyte differentiation and functioning (John *et al.*, 2010). Zinc deficiency limits thymulin production, reducing the number of circulating T-lymphocytes and the CD4 to CD8 ratio (Foster & Samman, 2012; John *et al.*, 2010).

T-lymphocyte signalling and maturation of CD4 and CD8 cells involves phosphorylation of $p56^{lck}$, a zinc-finger protein and lymphoid specific protein tyrosine kinase (Taylor & Giesbrecht, 2000). $p56^{lck}$ is involved in T-lymphocyte signal transduction through the maturation of thymocytes, the activation and proliferation of mature thymocytes, and cell selection and survival (Weil & Veillette, 1996). Cells over-expressing $p56^{lck}$ are hypersensitive to apoptosis, whereas under-expressing cells are resistant to apoptosis (Di Somma, Nuti, Telford, & Baldari, 1995). Dietary zinc deficiency is associated with elevated levels of splenic T-lymphocyte $p56^{lck}$, and this may contribute to excessive apoptosis and incomplete maturation of T-lymphocytes in mice (Taylor & Giesbrecht, 2000). Additionally, Hosea *et al.* (2003) found that $p56^{lck}$ protein

levels were higher in ZD rats than ZC rats, and 1 to 2-fold greater in thymocytes than splenocytes.

Diabetes and the Immune System

Diabetes and metabolic syndrome are strongly associated with inflammation, but the pathogenesis of these disorders is due to a chronic activation of the innate immune system (Calle & Fernandez, 2012; Fessler, Rudel, & Brown, 2009). Innate immune cells express pattern-recognition receptors which recognize pathogens, activating nuclear factor-kappa-β (NF-κβ) signalling pathways, stimulating a pro-inflammatory response (Calle & Fernandez, 2012). Activation of the innate immune system leads to atherosclerosis, insulin resistance and obesity (Fessler, Rudel, & Brown, 2009). It has been proposed that obesity activates the immune system by adipocyte organelle dysfunction (mitochondria and endoplasmic reticulum) and adipose tissue hypoxia (Calle & Fernandez, 2012). Obesity, and an increase in adipose tissue, leads to lipolysis, hyperlipidemia, and increased levels of cellular free fatty acids, which in addition to hyperglycemia, leads to increased oxidative stress and immune activation (Calle & Fernandez, 2012). Adipose tissue hypoxia is caused by hypertrophic adipose tissue and poor vascularisation (Calle & Fernandez, 2012). Hypoxia signals the activation of genes that contribute to angiogenesis, altered glucose metabolism, enhanced leptin secretion, cellular stress, and inflammation (Calle & Fernandez, 2012). Therefore, the innate immune system plays a significant role in the development of type 2 diabetes.

Diabetes also alters immune cell populations and the immune response. In diabetes-prone BioBreeding rats which spontaneously develop autoimmune diabetes,

CD90 cells have a shorter lifespan and undergo increased apoptosis, and thus there are fewer mature T-lymphocytes (Iwakoshi *et al.*, 1998). Therefore, diabetes alters immune cell proportions which may lead to the development of diabetes complications and co-morbidities.

Zinc, Diabetes, and the Immune System

Zinc is also involved in diabetes and immune dysfunction by helping to regulate apoptosis and cell death. Kolenko *et al.* (2001) showed that zinc chelation induces caspase-3 activation resulting in DNA fragmentation and apoptosis in T-lymphocytes. Apoptosis is pivotal to a variety of physiological processes but excessive apoptosis, especially in T-lymphocytes, contributes to the progression of many diseases such as cancer and diabetes (Feng, Li, Guan, Franklin, & Costello, 2008; Weissgarten *et al.*, 2002).

In diabetes, there is an increase in circulating free fatty acids which leads to a constant activation of the immune system, activating toll-like receptors (TLRs) on macrophages and adipocytes, increasing pro-inflammatory cytokines, and contributing to insulin resistance and immune dysfunction, further exacerbating diabetes (Bremer, Devaraj, Afify, & Jialal, 2011; Jialal, Huet, Kaur, Chien, & Devaraj, 2012).

Zinc deficiency exacerbates the complications of diabetes by disrupting the balance of cytokines, DNA repair enzymes, signalling systems (ie. insulin signalling) and zinc transporters, causing further impairment in immune system functioning (John *et al.*, 2010; Kanoni *et al.*, 2011; Sun, van Dam, Willett, & Hu, 2009). Zinc deficiency is associated with type 1 diabetes by the destruction of pancreatic β-cells through T-

lymphocyte-mediated cytotoxicity and cytokine-induced cell death (Jansen, Karges, & Rink, 2009). Cytokines have the ability to sensitize β-cells to T-lymphocyte mediated destruction through activation of the Fas receptor on the β-cell (Foster & Samman, 2012). Therefore, zinc deficiency exacerbates both diabetes and immune dysfunction.

Zinc, Inflammation, and Diabetes

Many of the symptoms of zinc deficiency and diabetes are related to an increase in inflammation (Fessler, Rudel, & Brown, 2009; John *et al.*, 2010). The immune system produces an inflammatory response to deal with stress or pathogens. The presence of chronic low-grade inflammation, and concomitant mild zinc deficiency, is a central feature of diabetes (Bremer, Devaraj, Afify, & Jialal, 2011; Foster & Samman, 2012; Suganami *et al.*, 2007). A characteristic of inflammatory disorders is the switch from cellular (Th1) to a less pro-inflammatory humoral (Th2) immune response resulting in an imbalance of cytokines; there is an increase in pro-inflammatory cytokines such as monocyte chemoattractant protein-1 (MCP-1) and TNF-α, and a down regulation of anti-inflammatory cytokines such as adiponectin (DiPiro, 1997; Lu, Xin, Tang, & Shao, 2012; Suganami *et al.*, 2007). Many of the signs of type 2 diabetes are caused by chronic inflammation and increased pro-inflammatory cytokines (Calle & Fernandez, 2012).

Jialal *et al.* (2012) found that circulating levels of pro-inflammatory cytokines and surface expression of TLRs (TLR-2, TLR-4) on monocytes are increased in patients with metabolic syndrome. TLRs are activated by lipids and induce pro-inflammatory cytokine production in macrophages and adipocytes (Calle & Fernandez, 2012; Suganami *et al.*, 2007). Calle & Fernandez (2012) found that increased adipose tissue mass can lead to a

constant activation of the innate immune system, through the activation of NFκB signalling pathways and insulin resistance.

Suppressor of cytokine signalling (SOCS)-3 is a member of the SOCS family which provides a negative feedback mechanism to reduce cytokine signalling (Emanuelli *et al.*, 2001). SOCS-3 can be rapidly induced by insulin, cytokines (especially TNF-α and IL-6), and fatty acids, reducing leptin sensitivity and inhibiting insulin signalling through a down-regulation of the the janus kinase (JAK)/signal transducer and activator of transcription (STAT) pathway (Emanuelli *et al.*, 2001; Munzberg & Myers, 2005; O'Connor, Sherry, Guest, & Freund, 2007; Palanivel *et al.*, 2012). Obesity elevates adipose tissue levels of SOCS-3 contributing to insulin resistance and glucose intolerance (Palanivel *et al.*, 2012). SOCS-3 over expression in adipose tissue leads to impaired insulin signalling and decreased insulin-stimulated glucose uptake in adipocytes (Shi *et al*., 2006). In *db/db* mice, SOCS-3 is elevated in adipose tissue, contributing to insulin resistance and reduced adiponectin production, via inhibition of JAK/STAT pathway (Kanatani *et al.*, 2007).

Haptoglobin is an acute phase protein and a circulating inflammatory marker that is synthesized primarily in the liver (Sadrzadeh & Bozorgmehr, 2004). The production of haptoglobin is increased by insulin and cytokines (Sadrzadeh & Bozorgmehr, 2004). Haptoglobin is also an antioxidant, an immunosurpressor of lymphocyte function, and a modulator of T-lymphocyte cytokine production and balance (Sadrzadeh & Bozorgmehr, 2004). Elevated haptoglobin levels are an indicator of inflammation (Sadrzadeh & Bozorgmehr, 2004) and are present in diabetes (Mohieldein, Alzohairy, Hasan, & Khan, 2012; Suzuki *et al.*, 2009).

MCP-1 is a cytokine produced by inflammatory cells and its expression is enhanced in pro-inflammatory responses (Kolattukudy & Niu, 2012). Increased expression of MCP-1 in adipose tissue recruits macrophages and contributes to adipose tissue inflammation and insulin resistance (Kolattukudy & Niu, 2012). Increased circulating levels of MCP-1 are found in obesity, type 2 diabetes, and insulin resistance (Kolattukudy & Niu, 2012). Bremer *et al.* (2011) found that individuals with metabolic syndrome have significantly higher adipose tissue MCP-1, C-reactive protein (CRP) (an acute phase inflammatory protein), pro-inflammatory cytokines, and an increase in macrophage infiltration, of adipose tissue compared to individuals without metabolic syndrome. Increased circulating CRP levels are associated with altered innate immune response and diabetes (Marques-Vidal *et al.*, 2012). Marques-Vidal *et al.* (2012) found plasma levels of pro-inflammatory cytokines such as TNF-α and IL-6, and CRP to be positively and significantly associated with levels of fasting plasma glucose and insulin, and insulin resistance. Insulin resistance is a prominent feature of type 2 diabetes and is in part due to an increase in inflammatory responses, endoplasmic reticulum stress, and mitochondrial dysfunction, which can impair the insulin signalling pathway by reducing the binding of insulin to the insulin receptor subsrate-1 (IRS-1) (Yu, Li, Ling, & Jin, 2012).

Chronic inflammation has been associated with altered zinc concentrations, and low serum zinc is evident in diabetes (Capdor, Foster, Petocz, & Samman, 2012; Chausmer, 1998; Seet *et al.*, 2011). Zinc is involved in inflammation by influencing the production and balance of Th1 to Th2 cytokines, TLR expression, and by controlling the metabolism of adipose tissue (Fessler, Rudel, & Brown, 2009; Foster & Samman, 2012;

John *et al.*, 2010). Low dietary zinc intakes (<7 mg/d for women, <9.5 mg/d for men) in overweight and obese adults have been found to up-regulate RNA gene transcription and expression of inflammatory cytokines, IL-1α, IL-1β, and IL-6, in peripheral blood mononuclear cells compared to obese adults with adequate zinc intakes (Costarelli *et al.*, 2010). Therefore, altered zinc homeostasis, which is present in diabetes, contributes to inflammation and immune dysfunction.

Obesity, which is common in type 2 diabetes, alters levels of pro- and anti-inflammatory adipokines (leptin and adiponectin), acute phase proteins such as haptoglobin, and zinc transporter expression (Bremer, Devaraj, Afify, & Jialal, 2011; Sadrzadeh & Bozorgmehr, 2004; Smidt *et al.*, 2007). Smidt *et al.* (2007) found lower levels of zinc transporters in visceral compared to subcutaneous adipose tissue, and in obese versus lean humans. Pro-inflammatory cytokines down-regulate zinc transporters, especially in islet cells, where ZnT8 is down-regulated 12-fold by cytokines (Egefjord *et al.*, 2009). Lu *et al.* (2012) found that zinc supplementation of 95 mg Zn/kg diet to Sprague-Dawley rats, decreased MCP-1 secretion in bronchoalveolar lavage fluid and elevated lung IFN-γ mRNA, improving the balance of Th1 to Th2 cytokines. Zinc supplementation has been shown to improve immunity (Sazawal *et al.*, 1997), reduce inflammation (Lu *et al.*, 2012), and may improve glycemic control (Simon & Taylor, 2001) and insulin sensitivity and signalling (Huber & Gershoff, 1973), improving the signs of diabetes. Although, high extracellular zinc (>100 μM) has been shown to stimulate cytokine production in primary mononuclear cells and increase inflammation in healthy adult humans (Foster & Samman, 2012). Therefore, it is the balance between zinc deficiency and toxicity that is essential for optimal health and more research is needed to

determine the appropriate dosing of zinc supplementation that can have the greatest benefits to individuals with diabetes.

Rodent Models of Type 2 Diabetes

The rat models of type 2 diabetes mellitus include the Goto-Kakizaki rat, Ostsuka Long-Evans Tokushima fatty rat, Spontaneously Diabetic Torii (SDT) rat, SDT fatty rat, Wistar fatty rat, and the Zucker diabetic fatty (ZDF) rat (Yokoi *et al.*, 2013). The best animal model to use is one that displays signs that are the most similar to that of the human disease (Siwy *et al.*, 2012). ZDF rats are a model of obesity which develop type 2 diabetes due to early onset β-cell loss and hyperglycemia (Lee *et al.*, 2010). At 6 to 10 weeks, male ZDF rats develop nonfasting hyperglycemia with blood glucose levels of approximately 20 mmol/L (Charles River, 2012; Kumar, Singh, Vasudeva, & Sharma, 2012; Yokoi *et al.*, 2013). Therefore, ZDF rats spontaneously develop type 2 diabetes within a short period of time. The other rat models are not as suitable for the study of type 2 diabetes since they lack some of the signs of diabetes or are not commercially available. A rat model is better for the study of zinc status and type 2 diabetes than a mouse model since it is a larger animal and can provide more tissue per animal allowing for multiple assessments and a more holistic picture of *in vivo* changes. Therefore, the ZDF rat is the best and most widely used animal model for studying type 2 diabetes with obesity (Paulsen, Vrang, Larsen, Larsen, & Jelsing, 2010; Yokoi *et al.*, 2013).

Dietary Zinc Levels for Producing Zinc Deficiency and Zinc Supplementation in Rodent Models

Varying levels of dietary zinc can be used to develop zinc deficiency and supplementation in animals. Hosea *et al.* (2003) used <1 mg Zn/kg diet to examine the effect of zinc deficiency on immune cells in weanling Sprague-Dawley rats. Less than 1 mg Zn/kg diet led to a reduction in feed intake and body weight within 3 weeks compared to rats fed zinc control. Femur zinc concentrations and recent thymic emigrants ($CD90^{+}$) were reduced accordingly with dietary zinc restriction. Therefore, <1 mg Zn/kg diet produces overt zinc deficiency and reduces growth in weanling rats. Simon and Taylor (2001) used 3 (MZD) and 300 (ZS) mg Zn/kg diet to assess the effects of dietary zinc on gylcemic control in *db/db* mice. Both 3 and 300 mg Zn/kg diet slightly reduced body weight but there was no effect of zinc on fat pad mass. Three mg Zn/kg diet did not affect pancreatic zinc concentrations but ZS of 300 mg Zn/kg diet was able to restore pancreatic zinc to the level of lean mice (Simon & Taylor, 2001). Petroulakis (2000) also assessed the effects of ZD (5 mg Zn/kg diet) in Zucker rats and discovered that ZD reduced femur zinc in both lean and Zucker rats, but did not affect serum insulin or glucose concentrations among dietary zinc groups for *fa/fa* Zucker rats. Thus, 5 mg Zn/kg diet is not low enough to produce changes in glycemic control in a non-diabetic insulin-resistant model while 1 mg Zn/kg diet is too extreme and results in anorexia. Furthermore, Simon and Taylor (2001) found that ZS reduced serum glucose concentrations compared to MZD in *db/db* mice. Femur zinc concentrations were elevated by ZS but only slightly reduced by MZD in mice (Simon & Taylor, 2001). In addition, femur calcium and phosphorus, and kidney and liver copper concentrations were not altered by dietary zinc

intake (Simon & Taylor, 2001). Therefore, 3 mg Zn/kg diet was able to achieve marginal zinc deficiency, and 300 mg Zn/kg diet was able to achieve zinc supplementation, in weanling *db/db* mice without any adverse effects of severe zinc deficiency or toxicity. Similarly, Tallman and Taylor (2003) found that C57BL/6J mice fed ZD (3 mg Zn/kg diet) had reduced femur zinc concentrations but there was no difference in body weight among ZD, ZC (30 mg Zn/kg diet), or ZS (150 mg Zn/kg diet). Dietary zinc did not affect serum glucose, insulin or leptin (Tallman & Taylor, 2003) suggesting that ZS of 150 mg Zn/kg diet is not high enough to produce beneficial changes in metabolic function in weanling C57BL/6J mice. Furthermore, Petroulakis (2000) found that ZS of 150 mg Zn/kg diet was not high enough to affect femur zinc concentrations (a marker of zinc status) in both lean and *fa/fa* Zucker rats or attenuate hyperinsulinemia in *fa/fa* Zucker rats. Therefore, these data indicates that ZS of 150 mg Zn/kg diet is not high enough to evaluate tissue zinc concentrations or ameliorate diabetes mellitus parameters, and that 300 mg Zn/kg diet is a better amount to use.

High dose zinc supplementation has been associated with some adverse effects. Jamieson (2005) fed weanling Sprague-Dawley rats ZS (300 mg Zn/kg diet) to prevent lead toxicity and measured mineral status in tissues (kidney, liver, intestine and bone). Jamieson (2005) found that tissue zinc concentrations reflected dietary zinc intake and that ZS impaired copper status but did not cause anemia in Sprague-Dawley rats. Therefore, high dietary zinc intake may alter copper status without disrupting health status. Although, high doses (about 120 mg Zn/day) of zinc in humans, in combination with low copper intake, can lead to severe neutropenia and anemia which can be reversed by normalizing zinc intake (Plum, Rink, & Haase, 2010). Zinc supplementation of 50

mg/d in adult females for 10 weeks reduced iron and copper status (Yadrick, Kenney, & Winterfeldt, 1989). Therefore, it appears that ZS of 300 mg Zn/kg is a safe amount to prevent the risk of developing other mineral deficiencies associated with high dose zinc supplementation. In conclusion, 3 mg Zn/kg diet and 300 mg Zn/kg diet are the most appropriate amounts of dietary zinc to produce changes in zinc status without adverse effects.

Current State of Knowledge

The relationships among zinc status, type 2 diabetes, and immune dysfunction are not clear. Some researchers are assessing the relationship between zinc and diabetes (Gunasekara, Hettiarachchi, Liyanage, & Lekamwasam, 2011; Ott & Shay, 2001; Petroulakis, 2000; Simon & Taylor, 2001; Sondergaard, Stoltenberg, Doering, Flyvbjerg, & Rungby, 2006; Sun, van Dam, Willett, & Hu, 2009), while others are attempting to bridge the gap between type 2 diabetes and inflammation/immune system functioning by looking at obesity and metabolic syndrome (Bremer, Devaraj, Afify, & Jialal, 2011; Calle & Fernandez, 2012; de Heredia, Gomez-Martinez, & Marcos, 2012; Fessler, Rudel, & Brown, 2009; Jialal, Huet, Kaur, Chien, & Devaraj, 2012). No studies to date have determined the relationships among zinc supplementation, diabetes, and immune system functioning. Zinc has been shown to lower blood glucose levels and play an essential role in the production, storage, and secretion of insulin from pancreatic islet cells (Kanoni *et al.*, 2011; Sun, van Dam, Willett, & Hu, 2009). Zinc deficiency and diabetes have similar symptoms which are associated with an increase in inflammation (Fessler, Rudel, & Brown, 2009; John *et al.*, 2010). Inflammation alters immune functioning, and immune dysfunction contributes to inflammation (Suganami *et al.*, 2007). Zinc is known to play a

role in both the innate and adaptive immune system responses and to alter cytokine levels (John *et al.*, 2010; Sazawal *et al.*, 1997). Previous studies have assessed the role of zinc in relation to diabetes (Basuki, Hiromura, & Sakurai, 2007; Brender *et al.*, 2010; Lemaire *et al.*, 2009; Simon & Taylor, 2001; Sun, van Dam, Willett, & Hu, 2009; Tang & Shay, 2001) or diabetes and inflammation/immune system functioning (Bremer, Devaraj, Afify, & Jialal, 2011; Calle & Fernandez, 2012; de Heredia, Gomez-Martinez, & Marcos, 2012; Fessler, Rudel, & Brown, 2009; Jialal, Huet, Kaur, Chien, & Devaraj, 2012; Kolattukudy & Niu, 2012; Marques-Vidal *et al.*, 2012; Suganami *et al.*, 2007), but no studies to date have combined both in a diabetes model. Some of the suggested mechanisms for zinc improving both diabetes and the immune system are through zinc acting as an insulin mimetic, reducing pro-inflammatory cytokines, and improving immune functioning (Bao, Prasad, Beck, & Godmere, 2003; Basuki, Hiromura, & Sakurai, 2007; Tang & Shay, 2001; Wiernsperger & Rapin, 2010). Therefore, the present study was performed to better understand the relationship among zinc, diabetes, and the immune system. This study was a dietary intervention which compared the effects of marginal zinc deficiency and zinc supplementation in a diabetic animal model and assessed the effects of zinc on both the parameters of diabetes and the immune system.

II. HYPOTHESES

1. Zinc supplementation improves glycemic control and insulin sensitivity by reducing inflammation. Marginal zinc deficiency will worsen glycemic control and insulin sensitivity by enhancing inflammation.
    - Zinc supplementation reduces pro-inflammatory cytokines and inflammatory markers, while the converse happens with marginal zinc deficiency.
    - Zinc supplementation reduces circulating free fatty acids and improves the blood lipid profile, reducing inflammation and insulin resistance, and improving islet cell function. The converse occurs in marginal zinc deficiency.
2. Zinc supplementation improves immune functioning by promoting T-lymphocyte maturation and the balance of Th1 and Th2 cytokine secretion. Marginal zinc deficiency has the opposite effect on immune function.

III. OBJECTIVES

- To determine if zinc supplementation improves the parameters of diabetes and if marginal zinc deficiency worsens diabetes by assessment of:
    - Obesity (body weight, fat pad mass, fat pad/body weight ratio, body composition).
    - Hyperglycemia (fasting serum glucose by spectrometric assay), hyperinsulinemia (fasting serum insulin by electrochemiluminescence), and glucose tolerance (OGTT).
    - Insulin resistance and beta-cell function (homeostasis model assessment [HOMA] of insulin resistance [IR] and beta-cell function [BCF], insulin sensitivity index [ISI]).
    - Insulin storage and β-cell hypertrophy in pancreatic islet cells (pancreatic islet insulin immunostaining).
    - Hyperlipidemia (fasting serum blood lipid profile by autoanalyzer, serum free fatty acids by spectrometric kit assay).
    - Hepatic steatosis (liver mass, total liver lipid concentrations).
    - Renal function (serum creatinine and urea, and urinary creatinine, urea, and glucose).
    - Inflammation (serum haptoglobin by spectrometric assay, serum MCP-1 by electrochemiluminescence, serum cytokines [TNF-α, IL-6, IL-1β, and IFN-γ] by muiltiplex assay, adipocyte MCP-1, SOCS-3, and TNF-α by Western blotting).
    - Bone structure and density (dual-energy X-ray absorptiometry [DEXA]).

- Zinc status and zincuria (pancreas, epididymal fat, liver, serum, femur, and urine zinc by inductively coupled plasma-optical emission spectrometry [ICP-OES] or atomic absorption spectrophotometry [AAS]), and zinc transporters in adipose tissue (ZnT3 by Western blotting).

- To determine if zinc supplementation and marginal zinc deficiency affects the immune cell proportions and cytokine secretion of immune cells isolated from the mesenteric lymph nodes:
  - Proportions of T-lymphocytes ($CD3^+$, $CD4^+$, $CD8^+$), B-lymphocytes ($CD45RC^+$), immature ($CD45RC^+$ $CD90^+$) and mature ($CD45RC^+$ $CD90^-$) B-lymphocytes, monocytes ($CD3^-CD4^+$), and natural killer cells ($CD3^-$ $CD8^+$) by flow cytometry.
  - Maturation of T-lymphocytes evaluated by recent ($CD90^+$) and late (mature) ($CD3^+$ $CD90^-$ $CD45RC^+$) thymic emigrants by flow cytometry.
  - Activated ($CD3^+$ $CD4^+$ $CD25^+$) and unactivated ($CD3^+$ $CD4^+$ $CD25^-$) helper and activated ($CD3^+$ $CD8a^+$ $CD25^+$) and unactivated ($CD3^+$ $CD8a^+$ $CD25^-$) cytotoxic T-lymphocytes, and naive ($CD3^+$ $CD45RC^+$ $CD4^+$) and memory ($CD3^+$ $CD45RC^-$ $CD4^+$) helper T-lymphocytes by flow cytometry: Th1 and Th2 cytokine secretion (IFN-γ, TNF-α, IL-6, and IL-1β from Concanavalin A stimulated or unstimulated cells using a multiplex assay).

IV. MATERIALS AND METHODS

Study Design

The study was an 8 week dietary intervention study using 40 male ZDF and 10 male lean (+/?) control ZDF rats (Charles River, St. Constant, PQ). The ZDF rat has a mutation in the leptin receptor, impairing leptin's signalling and appetite suppressing effects, leading to insulin resistance and obesity (Charles River, 2012; Leonard, Watson, Loomes, Phillips, & Cooper, 2005). These rats exhibit obesity, hyperinsulinemia, hyperglycemia, and insulin resistance beginning at 6 weeks of age and are a well known animal model for type 2 diabetes (Charles River, 2012; Leonard, Watson, Loomes, Phillips, & Cooper, 2005). Male ZDF rats exhibit insulin resistance and non-fasting blood glucose levels of 14 mmol/L by 8 weeks of age and >28 mmol/L by 12 weeks of age, at which time they are considered to have overt diabetes (Charles River, 2012). In male ZDF rats, a macronutrient composition of 27% of energy from protein, 17% of energy from fat, and 56% of energy from carbohydrates (Purina # 5008) induces programmed and consistent type 2 diabetes by 12 weeks of age (Charles River, 2012).

Animals and Diets

The animals arrived at 5 weeks of age and were acclimatized for a minimum of 1 week during which time they were fed the control diet (Figure 1). There was a total of 5 groups of rats (10 rats/group) and ZDF rats were randomly assigned to 1 of the 3 diets containing different levels of zinc to determine the physiological effects of varying zinc intake: marginal zinc deficiency (MZD, 4 mg Zn/kg Diet), zinc control (ZC, 30 mg Zn/kg diet as per AIN-93G), and zinc supplemented (ZS, 300 mg Zn/kg diet) (Table 2). The

remaining group of ZDF rats were a pair-weighed (PW) group in which PW rats were matched to an individual MZD rat and fed the specific amount of ZC diet to maintain a similar body weight as the paired MZD rat. The PW group acted as a control for all other groups to see if changes were due to diet composition or a reduction in body weight (since food intake decreases with zinc deficiency leading to weight loss and weight loss is known to improve diabetes parameters). Although, none of the PW rats were feed restricted as body weight was similar across ZDF groups, and therefore, PW rats were not included in any analyses. The lean ZDF rats were fed the ZC diet and acted as a control to interpret changes in the diabetic and immune parameters for all of the ZDF groups.

Each diet contained 21% of energy from protein, 17% of energy from fat, 60% of energy from carbohydrates, and 5% (wt/wt) fibre. The diet formula contained dextrose, egg white, cellulose, zinc-free mineral mix, vitamin mix, choline bitartrate, biotin mix, zinc pre-mix (according to amount specified for each diet), and soybean oil. Egg white was used instead of casein since the content of zinc in casein is too high to achieve 4 mg Zn/kg diet of zinc in the MZD diet. The biotin mix was added since egg white contains avidin which binds biotin, reducing its availability.

All animals were housed singly in polycarbonate cages with bedding and stainless steel environmental enrichment items, at 21^{o}C on a 12 hour light-dark cycle. We feel confident that we were able to develop zinc deficiency while using polycarbonate cages instead of wire cages, since our lab was able to produce severe zinc deficiency with a <1 mg Zn/kg diet using polycarbonate cages with bedding in a previous study performed in using Sprague Dawley rats (unpublished data).

Table 2. Diet Formulation

	MZD 4 mg/kg[1]	**ZC** 30 mg/kg[1]	**ZS** 300 mg/kg[1]
Ingredients (g/kg)			
Dextrose	609	600	510
Egg White	212.5	212.5	212.5
Cellulose	50	50	50
Mineral Mix (AIN-93G-MN, zinc-free)	35	35	35
Vitamin Min (AIN-93-VX)	10	10	10
Choline Bitartrate	2.5	2.5	2.5
Biotin Mix[2]	10	10	10
Zinc Pre-mix[3]	1	10	100
Soybean Oil	70	70	70

[1]Zinc concentrations confirmed by atomic absorption spectrometry. MZD = 4 mg Zn/kg diet, ZC = 30 mg Zn/kg diet, ZS = 302 mg Zn/kg diet.
[2]Biotin mix was prepared using 200 mg biotin/kg dextrose.
[3]Zinc pre-mix was prepared using 5.775 g zinc carbonate/kg dextrose.

In a previous MSc. project, ZS of 150 mg Zn/kg diet in *fa/fa* Zucker rats was not high enough to elevate femur zinc stores, and a zinc deficient diet of 5 mg Zn/kg diet was not sufficient to lower serum zinc concentrations (Petroulakis, 2000). Also, there were no changes in body weight among dietary zinc groups (Petroulakis, 2000), suggesting that 5 mg Zn/kg diet does not induce growth retardation. Therefore, from previous studies

conducted in our lab, we felt that 3 mg Zn/kg diet and 300 mg Zn/kg diet would be suitable for developing marginal zinc deficiency and zinc supplementation, respectively, and to assess differences among ZDF groups without any adverse effects of severe zinc deficiency or zinc toxicity. We designed the study for MZD to be 3 mg Zn/kg diet but analysis showed 4 mg Zn/kg diet indicating a higher background level of zinc in the other diet ingredients than expected.

All animals received zinc-free, ddH_2O (confirmed by atomic absorption spectrometry to contain <0.1 mg/kg zinc) from polyethylene water bottles with stainless steel sipper tubes, and *ad libitum* food intake, except for the PW group. Animal care procedures were approved by the University of Manitoba Protocol Management and Review Committee and were conducted according to the Canadian Council on Animal Care guidelines (Canadian Council on Animal Care, 2013).

The animals were fed the experimental diets for 8 weeks, starting at 6 weeks of age and were euthanized at 14 weeks of age. The animals had a staggered start with 10 rats (8 ZDF, 2 lean) randomly assigned to each group and starting the diet each week for 5 consecutive weeks. Each group of 10 animals were divided into 2 groups and assigned to start experimental diets on Tuesday or Thursday of each week; 4 ZDF rats were randomly assigned to each group (MZD, ZC, ZS, PW) and 1 lean animal was assigned to ZC.

An overview of the study design and timeline is shown in Figure 1. Feed intake and weekly body weights were recorded. Fasting saphenous blood and urine samples were obtained at 0, 4, and 8 weeks during the study. During week 8, OGTT was

performed and body composition was assessed *in vivo*. At the end of the study, and after 12 hours of fasting, the animals were euthanized using carbon dioxide asphyxiation and decapitation to ensure death. Body weight was recorded prior to decapitation. Trunk blood was collected. Various tissues were dissected, weighed and stored appropriately for analysis. The following sections provide further details.

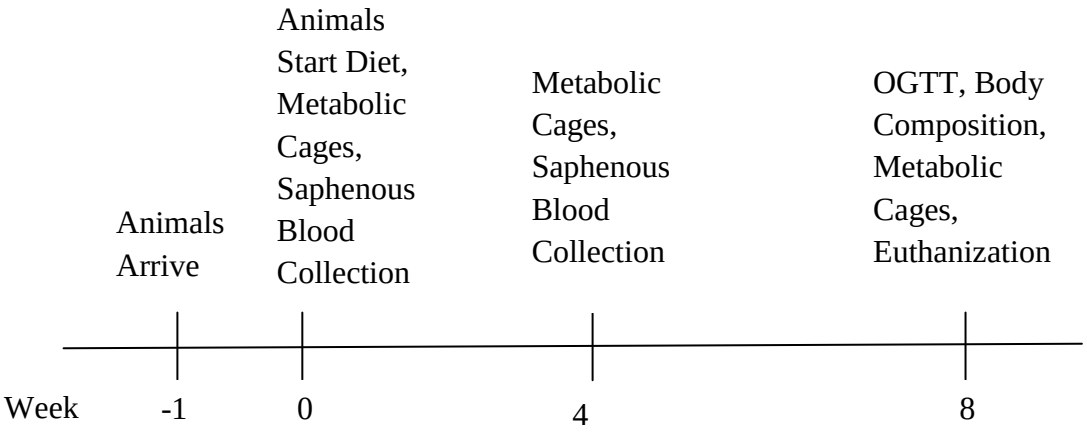


Figure 1. Study design. OGTT = Oral Glucose Tolerance Testing.

Feed Intake

Feed intake was recorded by weighing feed in and feed out 3 times per week, and calculating the difference.

Body Weight

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

Body Composition

Body composition was measured using an EchoMRI-700™ whole body Quantitative Magnetic Resonance (QMR) instrument (Echo Medical Systems, Houston, TX). This instrument uses the nuclear magnetic resonance properties of hydrogen atoms to fractionate signals originating from fat, lean tissue and free water (Taicher, Tinsley, Reiderman, & Heiman, 2003). Different signals are generated by fat, muscle, and tissue-free body fluids which allow for the quantification of each (Taicher, Tinsley, Reiderman, & Heiman, 2003). It has been reported that the EchoMRI-700™ instrument is very accurate and can measure the body composition of mice with a coefficient of variance ranging from 0.34 to 0.71% (Tinsley, Taicher, & Heiman, 2004). Animals were placed in a restraining tube, without the use of anaesthesia, and body composition was measured in less than 2 minutes. The EchoMRI-700™ was used to assess differences in body composition including fat mass, lean body mass, free water and total body water, in the animals at the end of the study (week 8). All measurements were done in triplicate.

Metabolic Cages: Urine and Saphenous Blood Collection

Metabolic cages were used for urine collection at 0, 4 and 8 weeks. Animals were fasted in metabolic cages for 5 hours with access to ddH_2O available *ad libitum.* Urine was collected in 30 mL plastic trace metal-free scintillation vials, weighed, aliquoted and stored at -80^oC until analysis. Urine was analyzed for creatinine, urea, glucose, and zinc.

Fasting saphenous blood samples were collected at 0, 4 and 8 weeks of the study after the rats had been in the metabolic cages for 5 hours. Blood was collected by shaving the hind limb of each animal and restraining each animal in a towel. The skin was pricked

using a sterile 22 gauge needle and blood was collected using microvette tubes (Sarstedt, Montreal, QC). One full microvette tube (~200 μL) was collected from each animal. Blood samples were allowed to coagulate for 30 minutes on ice, then centrifuged at 1000 g for 15 minutes at 4^{o}C. Serum was aliquoted, frozen and stored at -80^{o}C until analysis. Fasting saphenous blood samples were analyzed for glucose and insulin using colorimetric and electrochemiluminescence assays, respectively.

Oral Glucose Tolerance Testing (OGTT)

During week 8, OGTT was used to measure glucose clearance and glycemic control in response to an oral glucose dose. OGTTs help to determine insulin resistance and diagnose diabetes since fasting plasma glucose fails to diagnose 30% of cases of diabetes (Kumar, Singh, Vasudeva, & Sharma, 2012). After a 5 hour fast, the hind limbs were shaved and the rats were restrained in a towel to allow for the blood collection. Blood samples were collected from the saphenous vein of both hind limbs using microvette tubes and a 22 gauge needle. Baseline blood samples were taken prior to the animal receiving a dose of 2 g/kg (0.00286 mL/g body weight) of 70% glucose solution (in accordance with Gowda *et al.*, 2013) administered orally through a syringe (without a needle). Blood samples were collected at t=15, t=30, t=60, and t=120 minutes after glucose consumption. The amount of blood collected was 0.2 mL at all time points for a total of 1 mL of blood. Blood samples were allowed to coagulate on ice for about 30 minutes, then centrifuged at 1000 g for 15 minutes at 4^{o}C. The serum was collected, aliquoted, frozen and stored at -80^{o}C for analysis of glucose and insulin.

Tissue Collection

At the end of 8 weeks, rats were fasted overnight (12 hours) and euthanized using carbon dioxide asphyxiation and decapitation. Rats were weighed following carbon dioxide asphyxiation and trunk blood was collected after decapitation. A ruler was used to measure body length from the nose tip to the anus, and tail length from the anus to the tail tip. Trunk blood was collected in a 15 mL conical tube, allowed to coagulate, then centrifuged at 1000 g for 10 minutes at 4^{o}C. Tissues were dissected, weighed, immediately frozen in liquid nitrogen and stored at -80^{o}C for further analyses. The tissues collected and weighed were the pancreas, adipose (epididymal, peri-renal, mesenteric) tissue, liver, kidneys, thymus, and spleen. All fat pads were weighed but only the epididymal adipose tissue was kept and used for analyses since it was the main fat pad of interest (similar metabolic activity to visceral fat in humans). The mesenteric lymph nodes were collected and used immediately for cell culture isolation of immune cells and for *ex vivo* cytokine secretion, and analysis of the immune cell phenotypes using flow cytometry. A section of the pancreas was preserved in 10% phosphate-buffered formalin for sectioning. One excised femur underwent DEXA scanning, while the other was cleaned and frozen at -20^{o}C for analysis of zinc and other minerals.

Body Mass Index (BMI)

BMI is a measure of obesity and was calculated using the formula:

$$\text{BMI} = \text{body weight (g)}/\text{body length (cm)}^2$$

Lee's Index

Lee's index, a measure of adiposity (Cox, Laughton, & Powley, 1985), was calculated using the formula:

$$\text{Lee's Index} = \text{body weight (g)}^{0.33}/\text{body length (mm)}$$

Serum Biochemistry

Serum biochemistry was performed on all saphenous blood samples collected at 0, 4, and 8 weeks of the study, as well as trunk blood collected at time of euthanization, as summarized in Table 3.

Table 3. Serum Biochemistry

Blood Sample	Analysis
Fasting saphenous at 0, 4, and 8* weeks	Glucose, insulin
OGTT at 8 weeks	Glucose, insulin
Trunk blood	Glucose, insulin, zinc, haptoglobin, lipid profile, free fatty acids, cytokines (IL-6, TNF-α, IL-1β, IFN-γ, MCP-1)

* Fasting saphenous blood at 8 weeks was collected at t=0 of OGTT.

Glucose

Diabetes is characterized by high blood glucose levels (Chausmer, 1998; Islam & Loots du, 2007). Blood glucose (GLUC2, Roche Diagnostics, Indianapolis, IN, cat. #

04657527190) at termination was measured using the cobas c 111 clinical chemistry analyzer (Roche Diagnostics, Indianapolis, IN, USA). Blood glucose levels at weeks 0, 4, and 8 from saphenous blood samples were measured using a colorimetric assay, not by an autoanalyzer, since only small volumes of blood were collected from the saphenous vein at weeks 0, and 4, and 8 weeks and during OGTTs. All saphenous and trunk serum samples were analyzed using a colormetric glucose assay kit (Genzyme Diagnostics Glucose Reagent and Glucose Calibrator, cat. # 220-32, Charlottetown, PE, Canada; SEKISUI Diagnostics DC-CAL Calibrator, cat. # SE-035, North Vancouver, B.C., Canada).

Test Principle:

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

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

$$H_2O_2 + \text{hydroxybenzoate} + \text{4-aminoantipyrine} \xrightarrow{\text{Peroxidase}} \text{Quinoneimine Dye} + H_2O$$

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

Reagents:

1. Quality control solution was prepared according to instructions: 3 mL of DC-CAL Dilutent was added to DC-CAL Calibrator, swirled gently and allowed to sit for 30 minutes. DC-CAL quality control solution was mixed by inverting before use. The quality control calibration mean value for glucose was 9.7 mmol/L.
2. Glucose reagent was prepared according to instructions: 100 mL of double distilled water (ddH_2O) was added to the dry reagent, swirled gently, and allowed to sit for 5 minutes.
3. All solutions and samples were thawed and kept on ice (for all assays to follow).
4. The calibration solution (5 mmol/L) was used to prepare the standard curve by adding 50 µL of 5 mmol/L to 50 µL of ddH_2O to give a 2.5 mmol/L solution. This serial dilution was repeated until all concentrations for the standard curve were reached: blank (0), 0.625, 1.25, 2.5, and 5 mmol/L.

Procedure:

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

Insulin

Elevated levels of circulating insulin are associated with type 2 diabetes (Gual, Le Marchand-Brustel, & Tanti, 2005).

Test Principle:

Meso-Scale Discovery uses an antibody for a specific protein that is coated on one electrode or spot per well. The insulin assay is a sandwich immunoassay. Sample is added to each well and the insulin in the sample binds to the capture antibody on the electrode surface on the bottom of each well. The electrochemiluminescent labeled antibody is then added and binds to the insulin that was captured by the antibody, completing the sandwich. A read buffer is then added to provide the environment for electrochemiluminescence. A voltage is applied to the plate electrodes causing the labeled antibody to emit light. The intensity of light emitted corresponds to the amount of insulin in the sample.

Reagents:

1. Meso-Scale Discovery (MSD) Mouse/Rat Insulin Kit (cat. # K152BZC-2, Gaithersburg, MD).
2. Prepared Blocker A solution according to instructions using ultrapure water (cat. # 400000, lot. # 0430425-1, Cayman Medical Company, Ann Arbor, MI).
3. Prepared 1 L of wash buffer (0.05% Tween-phosphate buffered saline [PBS-T]) by adding 0.5 mL of Tween 20 (cat. # P5927-500 mL, Sigma Aldrich, St. Louis, MO) to 1 L of PBS (cat. # P5368, lot. # 081M82223, Sigma Aldrich, St. Louis, MO).

4. Prepared the highest concentration of the insulin calibrator by adding 10 µL of 5 ug/mL insulin calibrator to 990 µL of diluent 100.Vortexed to mix.
5. Prepared the remaining 6 controls by performing a 3-fold serial dilution using diluent 100 (ex. add 100 µL standard 1 to 200 µL diluent 100 to create standard 2). The last standard, standard 8 was a zero calibrator (diluent 100).
6. Prepared detection antibody by adding 50 µL of the 100× detection antibody solution to 4.95 mL of diluent 17 in a 15 mL conical tube, to create a 1× detection antibody solution and kept in the dark until use.
7. Prepared read buffer by adding 5 mL of 4× read buffer to 15 mL of ultrapure water in a 50 mL conical tube.

All samples were used undiluted.

Procedure:

Step 1:

- 150 µL of Blocker A was added to each well on the Meso-Scale Discovery plate using a multichannel pipette and reverse pipetting. Reverse pipetting is a technique used to ensure no bubble formation. A solution is aspirated into the pipette tip so that there is a greater volume of sample in the tip than will be delivered to the plate. This way there is always liquid that remains inside the tip after expelling the desired amount of liquid from the tip, preventing bubble formation. Reverse pipetting was used for all MSD assays.
- The plate was sealed with an adhesive plate seal and incubated for 1 hour at room temperature with vigorous shaking (300-1000 rpm, for all MSD assays).

Step 2:

- The plate was washed 3 times with 0.05% PBS-T and then the plate was tapped onto paper towel after the last wash to ensure all liquid was expelled from the wells (this method was used for all wash steps in MSD assays).
- 40 µL of the detection antibody was added to each well using a multichannel pipette and reverse pipetting.
- 10 µL of calibrator or sample (undiluted) was immediately added to each well in duplicate. The plate was sealed with an adhesive plate seal, covered in tinfoil to protect from light, and incubated at room temperature for 2 hours with vigorous shaking (300-1000 rpm).

Once the plate was incubating, ran the system test plate on the MSD Sector® Imager 2400 (Meso-Scale Discovery, Rockville, MD) to make sure it was working correctly (this was performed during this step for all MSD assays).

Step 3:

- The plate was washed 3 times.
- 150 µL of 1× read buffer was added to each well by reverse pipetting (very important to prevent bubble formation) and immediately read on the Sector® Imager 2400 using MSD Discovery Workbench® analysis software (this instrument and software was used for all MSD assays).

Standard curve range was from 0-50000 pg/mL. Only standards and samples with a CV of less than 20 were used (for all MSD assays). The lower limit of detection (LLOD)

was the calculated concentration of the signal that is 2.5 standard deviations over the zero calibrator. Percent recovery was calculated as the measured concentration divided by the concentration of the previous dilution (expected):

% Recovery = (measured × dilution factor) / (expected × 100)

Percent recovery for the standards was between 80–120 or the plate was redone (for all MSD assays).

Homestasis Model Assessment of Insulin Resistance (HOMA-IR)

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

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

Insulin values were converted from pg/mL to µU/mL (1 ng/mL = 172.5 pmol/L, 6.9 pmol/L = 1µU/mL).

Homestasis Model Assessment of Beta-Cell Function (HOMA-BCF)

HOMA-BCF uses fasting insulin and glucose concentrations to assess beta cell function (Matthews *et al.*, 1985). HOMA-BCF was calculated as follows:

$$\text{HOMA} - \text{BCF} = \frac{[20 \text{ x Fasting serum insulin } \left(\frac{\text{mU}}{\text{L}}\right)]}{[\text{Fasting serum glucose } \left(\frac{\text{mmol}}{\text{L}}\right) - \ 3.5]}$$

Insulin values for the calculations were expressed in international units (1 ng/mL = 172.5 pmol/L, pmol/l = 0.144 mU/L).

Insulin Sensitivity Index (ISI)

The ISI is highly correlated ($r = 0.73$, $P < 0.0001$) with the whole-body insulin sensitivity of a euglycemic insulin clamp (Matsuda & DeFronzo, 1999). The calculation is as follows:

$$\text{ISI} = 10000 \div \sqrt{[(\text{FPG} \times \text{FPI}) \times (\text{AUC OGTT Glucose} \times \text{AUC OGTT Insulin})]}$$

FPG = fasting plasma glucose, FPI = fasting plasma insulin, AUC = area under the curve.

Area-Under-the Curve (AUC)

AUC was calculated using the trapezoidal method (Purves, 1992):

$$AUC_{time1\text{-}0} = [(C_0 + C_1) \div 2] \times Time_{1\text{-}0}$$

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

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

Free Fatty Acids (FFA)

Elevated levels of circulating FFA are associated with obesity and type 2 diabetes (Jialal, Huet, Kaur, Chien, & Devaraj, 2012). The concentration of FFA in termination serum was measured using a spectrometric assay kit from Roche Diagnostics (Penzbery, Germany, cat. # 11 383 175 001).

Test Principle:

$$\text{Free fatty acids + CoA + ATP} \xrightarrow{\text{Acyl CS}} \text{Acyl-CoA + AMP + Pyrophosphate}$$

$$\text{Acyl-CoA + } O_2 \xrightarrow{\text{ACOD}} \text{Enoyl-CoA + } H_2O_2$$

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

Acyl-CoA, acyl-coenzyme A; ATP, adenosine-5'-triphosphate; Acyl CS, acyl-CoA synthetase; Enoyl-CoA, 2,3-enoyl-coenzyme-A; ACOD, acyl-CoA-oxidase; AMP, andeosine-5'-monophosphate; H_2O_2, hydrogen peroxide; TBHB, 2,4,6-tribromo-3-hydroxy-benzoic acid; 4-AA, 4-aminoantipyrine; POD, peroxidase

The amount of red dye produced is measured at 546 nm and is proportional to the amount of free fatty acids in the sample.

Reagents:

1. Reaction mixture A: 1 tablet of ATP, coenzyme A, acyl-CoA-synthetase, peroxidase, ascorbate oxidase, 4-aminoantipyrine and stabilizers, dissolved in 11 mL of potassium phosphate buffer, pH 7.8.
2. Reaction mixture B: 1 tablet of acyl-CoA-oxidase and stabilizers, dissolved in 0.6 mL ACOD dilution solution and stabilizers.
3. Solution 3: aqueous N-ethyl-maleinimide and stabilizers.

Procedure:

Reaction mixtures A and B were prepared at room temperature and allowed to dissolve for about 10 minutes, swirling occasionally to mix. Samples were thawed on ice

and vortexed to mix. Samples were used undiluted. Using a multichannel pipette, 200 µL of reaction mixture A was added to each well of a 96-well plate (NUNC^{TM}, Roskilde, Denmark, cat. # 167008). Next, 10 µL of a blank (ddH_2O), quality control (one sample was ran on each plate as an internal control), and sample were added to each well in triplicate. The reaction mixture A and samples were mixed by swirling the plate on the countertop for 30 seconds, then the plate was covered with the plate lid, and allowed to incubate at room temperature for 10 minutes. After the incubation period, 10 µL of solution C was added to each well. All bubbles we popped by gently blowing on the plate or by using a pipette tip. The plate was shaken on the microplate reader for 30 seconds using the mix function, and then the absorbency of each well was measured at 546 nm. This absorbency was labelled A1. The plate was removed and 10 µL of reaction mixture B was added to each well. Reaction mixture B and samples were mixed by swirling the plate on the countertop for 30 seconds, the plate was covered with the plate lid, and allowed to incubate at room temperature for 20 minutes. After the incubation period, all bubbles were popped, then plate was shaken on the microplate reader for 30 seconds and the absorbency was measured at 546 nm. This absorbency was labelled A2.

The concentrations of free fatty acids in the sample were calculated using the formula:

$$\Delta A = A2-A1$$

$$\text{free fatty acids (mM)} = 1.192 \times \Delta A$$

Lipid Profile

Dyslipidemia is common among people with diabetes and contributes to cardiovascular complications (Talayero & Sacks, 2011).Total cholesterol (TC,

Cholesterol generation 2, Roche Diagnostics, Indianapolis, IN, cat. # 04718917 190), high-density lipoprotein cholesterol (HDL-C, HDL-C plus 3rd generation, Roche Diagnostics, Indianapolis, IN, cat. # 05401488 190), low-density lipoprotein cholesterol (LDL-C, LDL-C plus 2nd generation, Roche Diagnostics, Indianapolis, IN, cat. # 05401682 190), and triglycerides (TG, Roche Diagnostics, Indianapolis, IN, cat. # 04657594 190) were measured using the cobas c 111 clinical chemistry analyzer (Roche Diagnostics, Indianapolis, IN, USA).

Haptoglobin

Haptoglobin is the predominant circulating acute phase protein in rats and is an assessment for chronic low grade inflammation associated with diabetes (Jelena *et al.*, 2013). Trunk serum was analyzed for haptoglobin using an enzymatic colorimetric assay kit from Tridelta Development Ltd. (Maynooth Ireland, cat # TP801).

Test Principle:

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

Reagents:

1. Reagent 1: Haemoglobin
2. Reagent 2: Chromogen

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

Procedure:

1. Standards and samples (undiluted) were vortexed and 7.5 µL of each was pipetted in duplicate onto a 96-well plate (NUNC™, Roskilde Denmark, cat# 167008).
2. Using a multi-channel pipette, 100 µL of reagent 1 was added to each well and samples were mixed by pipetting up and down 3 times.
3. Using a multi-channel pipette, 140 µL of reagent 2 was added to each well and samples were mixed again by pipetting up and down 3 times.
4. Samples were allowed to incubate for 5 minutes at room temperature during which all bubbles were popped by blowing on the plate and/or using a pipette tip.
5. After incubation, the absorbency of each well was immediately measured at 630 nm.

Note: Normal range for rat is 0.25-0.51 mg/mL, and the range for acute infection is 0.8-1.8 mg/mL.

Monocyte Chemoattractant Protein (MCP)-1

Increased circulating levels of MCP-1 are found in obesity, type 2 diabetes, and insulin resistance (Kolattukudy & Niu, 2012).

Test Principle:

Refer to insulin assay for test principle.

Reagents:

1. Meso-Scale Discovery Rat MCP-1Ultra-Sensitive Kit (cat. # K153AYC-1, Gaithersburg, MD)
2. Prepared1 L of wash buffer (0.05% Tween-phosphate buffered saline [PBS-T]) (refer to insulin assay).
3. Prepared calibration/standard curve which ranged from 0-40000 pg/mL.
    a. Prepared standard 1 by transferring 10 µL of 25× rat MCP-1 stock calibrator solution to 240 µL of diluent 6.
    b. The remaining 6 controls were prepared by performing a 4-fold serial dilution using diluent 6 (ex. add 50 µL standard 1 to 150 µL diluent 6 to create standard 2). The last standard, standard 8 was a zero calibrator (diluent 6).
4. The detection antibody was prepared by adding 60 µL of the 50× stock Anti-m/r MCP-1 detection antibody and 2.94 mL of diluent 5 and kept in the dark on ice until use.
5. 2× read buffer was prepared by adding 10 mL of 4x read buffer to 10 mL of ultrapure water in a 50 mL conical tube.

Procedure:

Step 1:

- 25 µL of diluent 6 was added to each well on the Meso-Scale Discovery plate using a multichannel pipette.

- The plate was sealed with an adhesive plate seal and incubated for 30 minutes at room temperature.

Step 2:

- Prepared serum samples by diluting ZDF serum 10 times (10 µL serum and 100 µL diluent 100) and lean ZDF 2 times (25 µL serum and 50 µL diluent 100) using diluent 100.

  Note: diluent 100 was used from a previous MSD kit (cat. # K152BZC-2).
- 25 µL of calibrator or sample was added to each well in duplicate.
- The plate was sealed with an adhesive plate seal and incubated at room temperature for 2 hours.

Step 3:

- The plate was washed 3 times.
- 25 µL of 1× detection antibody was added to each well using a multichannel pipette.
- The plate was sealed with an adhesive plate seal, covered with tinfoil, and incubated at room temperature for 2 hours.

Step 4:

- The plate was washed 3 times.
- 150 µL of 2× read buffer was added to each well and immediately read.

Creatinine and Urea

An elevated or reduced serum urea to creatinine ratio is a sign of kidney disorders and heart failure (Brisco *et al.*, 2013; Lindenfeld & Schrier, 2011), which are co-morbidities of diabetes. Serum creatinine (Roche Diagnostics, Indianapolis, IN, cat. # 05401755 190) and urea (urea/BUN, Roche Diagnostics, Indianapolis, IN, cat. # 04657616 190) were measured using the cobas c 111 clinical chemistry analyzer (Roche Diagnostics, Indianapolis, IN, USA).

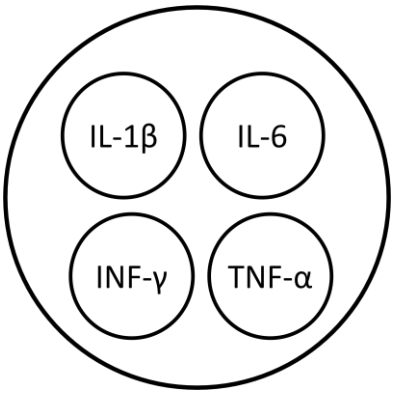
Cytokines (TNF-α, IL-6, IFN-γ, IL-1β)

Many of the signs of type 2 diabetes are caused by chronic inflammation and an imbalance in cytokine secretion (Calle & Fernandez, 2012).

Test Principle:

Refer to insulin assay for test principle.

Spot Layout:



Reagents:

1. Meso-Scale Discovery Custom Multiplex 4-Spot Mouse/Rat Cytokine Kit (cat. # 99906, Gaithersburg, MD)
2. Prepared1 L of wash buffer (0.05% Tween-phosphate buffered saline [PBS-T]) (refer to insulin assay).
3. Prepared cytokine calibrator combined working stock solution (1 µg/mL) by adding 10 µL of each 50 µg/mL individual cytokine stock (total of 40 µL) to 460 µL of diluent 6.
4. Prepared calibrator/standard curve which ranged from 0-40000 pg/mL.
    a. Prepared standard 1 by transferring 10 µL of the combined working stock solution to 240 µL of diluent 6.
    b. Prepared the remaining 6 controls by performing a 4-fold serial dilution using diluent 6 (ex. add 50 µL standard 1 to 150 µL diluent 6 to create standard 2). The last standard, standard 8 was a zero calibrator (diluent 6).
5. The detection antibody was prepared by adding 60 µL of the 50× stock detection antibody mix and 2.94 mL of diluent 5 and kept in the dark on ice until use.
6. 2x read buffer was prepared by adding 10 mL of 4× read buffer to 10 mL of ultrapure water in a 50 mL conical tube.

Procedure:

Procedure followed the same method as MCP-1. Samples were used undiluted.

One sample was run on each plate as an internal control. Total serum cytokine content was calculated by adjusting for total blood volume [58 mL blood volume/kg body weight (Olfert, Cross, & McWillam, 1993)].

Total Liver Lipid Concentration

Hepatic steatosis is common in type 2 diabetes and may precede or exacerbate hepatic insulin resistance (Roden, 2006; Schmid *et al.*, 2011). Total liver lipid was determined by a modified Folch method (Folch, Lees, & Sloane Stanley, 1957). A 2:1 chlorofom:methanol stock solution (300 mL choloroform: 150 mL methanol) was prepared. Using a glass graduated cylinder, 22 mL of chloroform:methanol solution was put into 50 mL glass tubes, capped, and placed on ice. Liver was removed from the -80^{o}C freezer and placed immediately on ice. Approximately 1 gram (exact weight was recorded to 3 decimal places) of liver from each rat was placed in the 50 mL tubes containing 22 mL of chloroform:methanol solution. The liver choloroform:methanol solution were homogenized for 20 seconds with a Polytron PT-MR 1600 E (Lucerne, Switzerland). The homogenizer was cleaned before and after each sample with a 50 mL tube containing about 20 mL of chloroform:methanol solution and homogenizing for 20 seconds. The homogenate (22 mL) was filtered through #1 Whatman filter paper (cat. # 1001-125, GE Healthcare UK Limited, Buckinghamshire, UK) into a 25 mL graduated cylinder. Next, 20% of the volume of the graduated cylinder was added as 0.73% NaCl (i.e. 20% of 22 mL = 4.4 mL; therefore, 4.4 mL of 0.73% NaCl was added to homogenate through the filter paper into the graduated cylinder; 0.73% NaCl = 0.73 g NaCl + 100 mL ddH_20). The cylinder was then stoppered and the mixture was inverted 5 times and left to separate overnight at room temperature in the fume hood. Additionally, 20 mL glass

scintillation vials were placed in the dessicator for use the next day (label vials and caps, remove cap from vial and place on bottom of dessicator beside vial). The next day, the glass scintillation vials were removed from the dessicator and weighed (including caps). The volume of the lower chloroform/lipid layer (approximately 12 mL) was recorded and the top layer was removed using a glass pipette. Then, 10 mL of the bottom (choloroform/lipid layer) was removed using a glass pipette and placed into the dried and weighed 20 mL glass scintillation vial. The chloroform was then evaporated under nitrogen at 30^{o}C with a N-EVAP III Nitrogen Evaporator (Organomation Associates, Inc., Berlin MA). After being dried under nitrogen, the 20 mL glass scintillation vials containing the lipid were placed in the dessicator and dried overnight (caps were removed and placed on bottom of dessicator beside vial). The following day, the vials were weighed (including caps) and the lipid content was calculated as follows:

$$\text{g lipid/g tissue} = \frac{(\text{dry weight + vial}) - (\text{dry weight vial})}{10 \text{ mL chloroform used}} \times \text{volume of chloroform layer}$$

Pancreatic Islet Insulin Immunostaining

In ZDF rats, pancreatic islet cell mass enlarges until 16 weeks of age to compensate for insulin resistance, and then it declines along with islet cell function (Paulsen *et al.*, 2010). As type 2 diabetes progresses, a reduction in islet cell mass is common leading to a decline in insulin production (Jansen, Karges, & Rink, 2009). The formalin-fixed pancreas tissue was sent to the Manitoba Tumor Bank (CancerCare, Winnipeg, MB) for processing into paraffin blocks and preparation of 6 μm cryosections of pancreatic tissue on positively charged slides. Insulin immunostaining was performed with insulin antibodies to visualize the islet cells in the tissue sections.

Test Principle:

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

Reagents:

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

Procedure:

The series of solvents used were in a staining station and the slides were placed in a holder so that up to 10 slides could be stained at the same time.

Step 1:

- Deparaffinized tissue sections by placing them in:

- Old xylene (cat. # Z3S-4, Fisher Scientific) for 15 minutes. Old xylene has been previously used for this step for up to 45 slides.
- New xylene for 15 minutes.

Step 2:

- Rehydrated tissue sections by placing slides in:
  - 100% ethanol for 3 minutes.
  - 100% ethanol for 3 minutes.
  - 95% ethanol for 3 minutes.
  - 70% ethanol for 3 minutes.
  - ddH_2O for 5 minutes.

Step 3:

- Endogenous peroxidase activity of tissues was blocked by placing slides in:
  - Fresh 3% H_2O_2 for 10 minutes.
  - 1× PBS until the next step.

Step 4:

- Staining:
  - Slides were removed from PBS and the bottom of the slides and around tissue sections were dried with a paper towel.
  - The PAP pen was used to draw circles around tissue sections to contain the next solutions placed on the tissue sections.
  - 250 µL of Background Buster was pipetted onto each tissue section.

- Slides were incubated in the incubation chamber (sealable container with damp paper towels lining the bottom) for 20 minutes.
- Background Buster was poured off and the bottom of the slides and around the tissue sections were dried with a paper towel.
- 250 µL of 1^{o} antibody (insulin antibody) was pipetted onto the tissue section farthest from slide label.
- 250 µL of 1× PBS was pipetted onto tissue section closest to slide label (to be used as the control).
- Slides were incubated in incubation chamber for 20 minutes.
- Slides were removed from the chamber and rinsed with 1× PBS.
- Slides were placed in a 1× PBS bath for 5 minutes.
- 250 µL of the 2^{o} antibody (HRP-labelled Strepavidin) was pipetted onto each tissue section.
- Slides were incubated in the incubation chamber for 10 minutes.
- Slides were rinsed with 1× PBS and placed in a fresh 1× PBS bath for 5 minutes.
- 250 µL of 3^{o} antibody (Mouse Link Rat Absorbed) was pipetted onto each tissue section.
- Slides were incubated in incubation chamber for 10 minutes.
- Slides were rinsed with 1× PBS and placed in a fresh PBS bath for 5 minutes.
- While slides were in last PBS bath, DAB solution was prepared in the fume hood.

        - Ex. For 9 slides (18 tissue sections), 5 mL of DAB solution was prepared by mixing 5 mL of DAB substrate with 5 drops of DAB in a small beaker.
        - DAB was kept protected from light at all times.
    - Slides were taken out of the PBS bath and 250 µL of DAB solution was pipetted onto each tissue section.
    - Slides were incubated in the fume hood for 5 minutes.
    - Any unreacted DAB was rinsed off with ddH_2O (using a squirt bottle).
    - Tissue sections were counterstained with Mayer's hematoxylin for 1 minute.
    - Excess hematoxylin was rinsed off by dipping slides in a beaker of tap water 3 times.

Step 5:

- Tissue sections were dehydrated by placing them in:
    - 70% ethanol for 3 minutes.
    - 95% ethanol for 3 minutes.
    - 100% ethanol for 3 minutes.
    - 100% ethanol for 3 minutes.

Step 6:

- Covered slides by:
    - Immersing slides in new xylene for 3 minutes.
    - Laid slides flat on paper towel and let excess xylene evaporate.

- Mounted cover slips onto slides by placing a drop of Permount onto the middle of the slide (not on tissue sections).
- Cover slides were positioned over the tissue sections in the middle of the slide and pushed down to remove air bubbles.
- Slides were allowed to dry overnight in the fume hood.

Islet cells were stained a brown/red colour while other cells remained purple/blue. Islet cells were visualized under an Evos™ XL Core microscope (Fisher Scientific Inc., Ottawa, ON). Images were taken at 4× objective magnification, and then islet cell size and number was quantified using ImageJ 1.42 software (National Institutes of Health, USA).

Urinary Creatinine, Urea, and Glucose

ZDF rats develop diabetes related kidney problems and progressive nephropathy over time (Baynes & Murray, 2009; Suzaki, Ozawa, & Kobori, 2006). Kidneys become hypertrophic with the onset of diabetes and diabetic nephropathy leads to renal damage and end-stage renal disease. (Forbes & Cooper, 2013; Suzaki, Ozawa, & Kobori, 2006). As diabetes progresses, hyperfiltration increases the amount of glucose and other metabolites excreted in the urine (Baynes & Murray, 2009; Forbes & Cooper, 2013). Urine creatinine (Roche Diagnostics, Indianapolis, IN, cat. # 05401755 190), urea (urea/BUN, Roche Diagnostics, Indianapolis, IN, cat. # 04657616 190), and glucose (Roche Diagnostics, Indianapolis, IN, cat. # 04657527 190) were measured using the cobas c 111 clinical chemistry analyzer (Roche Diagnostics, Indianapolis, IN, USA).

Inductively Couple Plasma-Optical Emission Spectrometry (ICP-OES) and Atomic Absorption Spectroscopy (AAS)

ICP-OES and AAS were used to determine zinc status and the effects of MZD and ZS on tissue zinc concentrations and other minerals. ICP-OES and AAS are analytical techniques that measure the absorption of light of free atoms to determine the concentration of elements and trace metals. Liquid samples (serum and urine) are diluted to appropriate concentrations, while solid samples (pancreas, adipose, femur, liver and diet) are digested using trace-element grade nitric acid then diluted. Ten gram diet samples from each diet were collected and stored in a 15 mL scintillation vial at -80^{o}C for analyses. Zinc and other mineral concentrations from each sample were determined using ICP-OES or AAS. ICP-OES was used for mineral quantification of the femur, liver, and pancreas. AAS has a lower detection range and was used for determination of zinc in adipose tissue, diet samples, serum, urine, and cell culture media.

ICP-OES

Tissue Preparation:

- Femur

Right femurs were removed from the animal carcass and cleaned of all skin and tissue. Wet weight was recorded. A dial calliper (Mitutoyo Mfg. Co, Ltd., Japan) was then used to measure (in triplicate) the femur's total length, width at knee joint, diaphysis, hip joint, head, and neck. Femurs were then placed in a drying oven at 85^{o}C and dried for 48 hours. Femurs were removed from the oven 5 at a time and dry weight

was recorded. Femurs were immediately placed into labelled trace element-free, disposable DigiPREP tubes (SCP Science, Baie d'Urfé, Quebec) and sealed with a cap.

- Liver

Liver samples were cut to about 1.5 g and exact wet weight was recorded. Liver samples were placed in a drying oven at 85^{o}C and dried for 48 hours. Dry weight was recorded and samples were immediately placed into labelled DigiPREP tubes and sealed with a cap.

- Pancreas

The entire pancreas from each animal (minus the section that was formalin-fixed for insulin immunostaining) was used and wet weight was recorded. Pancreas samples were placed in a drying oven at 85^{o}C and dried for 48 hours. Dry weight (dry pancreas plus liquid lipids) was recorded and samples were immediately placed into labelled DigiPREP tubes and sealed with a cap.

Sample Digestion:

Once all samples were dried, 4 mL of 70% trace-metal grade nitric acid was added to each tube. Tubes were capped and the sample was allowed to digest at room temperature for 1 hour. After 1 hour, caps were removed and DigiPREP disposable watch glasses were placed on top of the sample tubes. Samples were then placed into a DigiPREP Jr. block digestion system (SCP Science, Clark Graham, Baie D'Urfe, QC), heated at 85^{o}C and allowed to digest for 2 hours. After 2 hours, samples were removed and allowed to cool for 1 hour. Femur and liver were then diluted to 20 mL using ddH_2O.

Pancreas samples were diluted to 10 mL. Diluted samples were then filtered in acid washed glass funnels through Q5 filter paper (Fisher Scientific, Ottawa, ON, cat. # 09-790-2C). Bovine liver reference (0.5 g, National Institute of Standards and Technology, Gaithersburg, MD) was processed in triplicate as a quality control and 1 nitric acid blank (4 mL nitric acid and 16 mL ddH_2O) was included with each tissue and diet sample, except the pancreas nitric acid blank was diluted to 10 mL instead of 20 mL. Zinc concentrations were measured in all samples. In addition, copper and iron concentrations were measured in liver, and calcium and phosphorus was measured in femur. Samples were submitted to the Department of Animal Science at the University of Manitoba (Winnipeg, Manitoba) for ICP-OES analysis. Zinc, copper, iron, phosphorus and calcium were all measured using simultaneous inductively coupled plasma-optical emission spectrometry (Varian ICP, Model-VISTA-MPX, CCD, Mississauga, ON).

AAS

Sample Preparation:

- Epididymal Fat

About 1.5 grams of epididymal fat from each animal was used and exact wet weight was recorded. Epididymal fat samples were placed in a drying oven at 85^{o}C and dried for 48 hours. Solid dry epididymal tissue was weighed and recorded, and samples were immediately placed into labelled DigiPREP tubes and sealed with a cap.

- Diet samples

An appropriate amount of each diet was weighed (0.20 g of MZD diet, 0.10 g of ZC, and 0.05 g of ZS) and placed into labelled DigiPREP tubes and sealed with a cap.

- Serum

Serum samples from ZDF rats contained a high amount of fat, and therefore, all serum samples had to be digested. Two hundred microlitres of serum was aliquoted and placed into a microfuge tube. One millilitre of nitric acid was added to each sample and then samples were heated in a hot water bath for 1 hour. After 1 hour, samples were cooled and then diluted 5-fold with ddH_2O (1 mL of digested sample and 4 mL ddH_20).

- Urine

No digestion was needed. All week 0 and MZD ZDF urine samples were diluted 5-fold, while week 8 ZC ZDF, ZS ZDF, and lean rat urine samples were diluted 10-fold with ddH_2O.

- Cell Culture Media

No digestion or dilution was needed. Samples were run as is. AAS was used to measure levels of zinc in cell culture media to verify that a low-zinc environment was used during cell culture experiments.

Sample Digestion:

Once all epididymal fat and diet samples were prepared, 1 mL of 70% trace-metal grade nitric acid was added to each tube. Tubes were capped and the sample was allowed to digest at room temperature for 1 hour. After 1 hour, caps were removed and DigiPREP

disposable watch glasses were placed on top of the sample tubes. Samples were then placed into a DigiPREP Jr. block digestion system, heated to 85^{o}C and allowed to digest for 2 hours. After 2 hours, samples were removed and allowed to cool for 1 hour. Epididymal fat samples were then diluted to 5 mL using ddH_2O. MZD diet was diluted to 5 mL, while ZC and ZS diets were diluted to 15 mL and 50 mL, respectively. Bovine liver reference (0.150 g, National Institute of Standards and Technology, Gaithersburg, MD) was processed in triplicate as a quality control and 1 nitric acid blank (1 mL nitric acid and 4 mL ddH_2O) was included with each tissue and diet sample. Zinc concentrations were measured in each sample using AAS (Perkin Elmer 3100 flame atomic absorption spectrometer, Waltham, MA, USA). The light source was a zinc hollow cathode lamp with a 6 mA applied current. The gas flow was 1.5 L/min of acetylene and 4.5 L/min of compressed air to give a lean (hot) flame. The wavelength was achieved with a Czemy Turner monochromator and set at 214 nm and the absorbance was measured every 2 seconds. The standard curve was prepared using 1000 mg/kg zinc reference standard (Fisher Scientific, Ottawa, ON, cat. # SZ13-100), which was diluted to 100 mL with ddH_2O and 1 mL of nitric acid to achieve 0.1, 0.2, 0.5, 1, and 2 mg/kg. A nitric acid blank (1 mL nitric acid and 99 mL ddH_2O) was used as the blank (0) for the standard curve. Total serum content was calculated by adjusting for total blood volume [58 mL blood volume/kg body weight (Olfert, Cross, & McWillam, 1993)].

Dual-Energy X-Ray Absorptiometry (DEXA) Scan

Analysis of bone area, bone mineral content, and bone mineral density of femurs was performed using DEXA scanning (Hologic Discovery A [S/N 86814], Bedford, MA) at the Manitoba Institute of Child Health. Excised left femurs were placed in a plastic

water bath aligned to an anteroposterior position with 3 cm of water covering the femur (Kastl, Sommer, Klein, Hohenberger, & Engelke, 2002). Sub-region high-resolution mode was used (version 13.3.0.1:3).

Immune Cell Isolation and Cytokine Secretion

Zinc is required for the physiological functioning of the both the innate and adaptive immune systems, and especially for the development and maturation of T-lymphocytes (Jansen, Karges, & Rink, 2009). The first consequence of zinc deficiency is impaired immune function; levels of circulating cytokines (produced from lymphocytes, granulocytes, and platelets) fluctuate in response to altered zinc status before a change in plasma zinc concentration can be detected (Foster & Samman, 2012; Prasad, 2008). Zinc deficiency limits thymulin production, reducing the number of circulating T-lymphocytes and the CD4 to CD8 ratio (Foster & Samman, 2012; John *et al.*, 2010). Additionally, diabetes and metabolic syndrome are strongly associated with inflammation, and the pathogenesis of these disorders is due to a chronic activation of the innate immune system (Calle & Fernandez, 2012; Fessler, Rudel, & Brown, 2009). Mesenteric lymph nodes are embedded in adipose tissue and are likely to be affected by cytokines and adipokines secreted from adipose tissue. Mesenteric lymph nodes have a higher proportion of T-lymphocytes than other lymphoid organs like the spleen, in lean and obese rats (68 and 63% vs. 48 and 50% in lean and obese rats, respectively) (Blewett, Gerdung, Ruth, Proctor, & Field, 2009). Therefore, mesenteric lymph nodes were used for immune cell culture and cytokine secretion since we were interested mainly in T-lymphocyte function and cytokine secretion in relation to adipose tissue dysfunction in type 2 diabetes.

Cell Isolation

All instruments were sterilized overnight in 70% ethanol prior to use. The mesenteric lymph nodes were aseptically removed, and placed in a petri dish containing buffer 1. Buffer 1 was filter sterilized, had a pH of 7.4, and contained 100 mL of 10× Hank's buffered saline, 2.383 g HEPES, 40 mL charcoal dextran fetal bovine serum (charcoal dextran binds to zinc and other minerals in the fetal bovine serum thus reducing levels of zinc and other minerals, Invitrogen, cat. # 12676), 10 mL antibiotic/antimycotic (Gibco®, cat. # 15240-062) and enough ddH_2O to make 1 L of solution. The mesenteric lymph nodes were pressed through a nylon mesh screen using the rubber end of a sterile 10 mL syringe into a petri dish containing 10 mL of buffer 1. Using the same syringe, the cell suspension was transferred to a 50 mL conical tube. The petri dish was rinsed by adding 10 mL of buffer 1 and the cell suspension was transferred to the 50 mL conical tube. The rinse step was repeated for a total of 30 mL buffer 1 in the 50 mL conical tube. The samples were centrifuged at 300 g for 10 minutes at 4^{o}C to pellet cells. The supernatant was rapidly decanted and the cell pellets were re-suspended in 5 mL of cell culture medium.

Cell Counting and Viability

To determine the number of cells in each sample, 10 µL of the cell suspension was diluted with 490 µL of cell culture medium. To determine the proportion of live to dead cells, 10 µL of the diluted cell suspension was mixed with 10 µL of 0.4% Trypan Blue solution and allowed to stain for 1 minute. Stained cell samples (10 µL) were loaded onto a counting chamber slide and counted using the Countess® Cell Counter (Life

Technologies, Burlington, ON). The Countess® Cell Counter uses an image analysis algorithm to measure the average number of live, dead, and total cells in the sample with a measurement range from 1×10^4 to 1×10^7 cells/mL. Excess cell suspension (not needed for mitogen-stimulation or flow cytometry) was aliquoted into 1.5 mL microcentrifuge tubes, centrifuged at 10 392 g, for 10 minutes. The supernatant was rapidly decanted and cell pellet was stored at -80^{o}C.

Cytokine Secretion

Once the number of cells was determined, 1×10^6 cells were transferred to three 4 mL cell culture tubes. Cell culture medium was added to the 4 mL cell culture tubes to bring them up to a total volume of 2 mL. One sample from each animal was stimulated for 24 hours using 5 µg/mL Concanavalin A (a T-lymphocyte mitogen) to enhance cytokine secretion. Concanavalin A was made by dissolving 100 mg of Concanavalin A (MP Biomedical, cat. # 150710, lot. # MR28590) in 2 mL of cell culture medium to create a solution containing 50 mg/mL of Concanavalin A. The solution was diluted by taking 10 µL of 50 mg/mL Concanavalin A solution and adding it to 990 µL of cell culture medium. This gave a stock solution of 0.5 mg/mL. The final working solutions was created by removing 220 µL of the 0.5 mg/mL stock solution and adding it to 1780 µL of cell culture media for a final working solution of 5 µg/mL. There were a total of 3 tubes of cells from each animal which were incubated at 37^{o}C, 5% CO_2 for i) 0 hours unstimulated, ii) 24 hours unstimulated, or iii) 24 hours Concanavalin A-stimulated. Either immediately or following 24 hours of incubation, cells were pelleted by centrifuging at 300 g for 5 minutes at 4^{o}C. The supernatant was rapidly decanted into a 20 mL scintillation vial. A 100 µL aliquot was removed and stored in a 0.5 mL

microcentrifuge tube for analysis of cytokine secretion through a multiplex assay (see cytokine secretion assay). Both supernatant aliquots and vials were frozen and stored at -80^{o}C until analysis.

Flow Cytometry

Zinc deficiency is known to adversely affect the innate and adaptive immune systems, especially lymphocyte proliferation and T-lymphocyte function (John *et al.*, 2010). Zinc supplementation of 10 mg per day (20 mg during diarrhea) in children has been shown to improve cellular immunity by increasing T-lymphocytes, helper T-lymphocytes, and the CD4:CD8 ratio (Sazawal *et al.*, 1997). Therefore, flow cytometry was used to assess immune cell proportions as an indicator of immune function in ZDF rats.

Flow cytometry can be used to differentiate immune cell populations (Delude, 2005). Cells are stained using a fluorescent antigen-specific antibody that recognizes specific cell surface markers (Delude, 2005). A suspension of antibody-stained cells is drawn into the machine and passes through a laser beam one cell at a time (Delude, 2005). Light from the laser is reflected as forward scatter, which represents the size of the cell, and side-scattered light is proportional to the cell's granularity and internal structures (Delude, 2005). Cells can be labelled using direct or indirect labelling with fluorochromes (Delude, 2005). In direct labelling, a monoclonal antibody with a conjugated fluorochrome is used, while in indirect labelling, a fluorochrome conjugated secondary antibody is used to recognize the nonconjugated antigen specific antibody

(Delude, 2005). Each fluorochrome emits a different wavelength of light which is used to determine different characteristics and separate cells (Delude, 2005).

Samples of mesenteric lymph node immune cells from each animal were used for flow cytometry to quantify the proportions of each immune cell type. Samples of mesenteric lymph node cells from each animal were aliquoted into 2 tubes. Cells in tube #1 were stained with flourochrome-conjugated antibodies for CD3 (BD Biosciences, Mississauga, ON, cat. # 557030, lot. # 34320), CD4 (BD Biosciences, cat. # 554839, lot. # 30261), CD8a (BD Biosciences, cat. # 554856, lot. # 11170), and CD25 (BD Biosciences, cat. # 554866, lot. # 25044). Tube #1 allowed for the quantification of the total proportions of T-lymphocytes ($CD3^+$) natural killer cells ($CD3^-$ $CD8a^+$), monocytes/macrophages ($CD3^-$ $CD4^+$), cytotoxic T-lymphocytes ($CD3^+$ $CD8a^+$), helper T-lymphocytes ($CD3^+$ $CD4^+$), activated ($CD3^+$ $CD8a^+$ $CD25^+$) and unactivated ($CD3^+$ $CD8a^+$ $CD25^-$) cytotoxic T-lymphocytes, and activated ($CD3^+$ $CD4^+$ $CD25^+$) and unactivated ($CD3^+$ $CD4^+$ $CD25^+$) helper T-lymphocytes. Cells in tube #2 were stained with flourochrome-conjugated antibodies for CD3 (BD Biosciences, cat. # 557030, lot. # 34320), CD4 (BD Biosciences, cat. # 554839, lot. # 30261), CD90 (BD Biosciences, cat. # 554897, lot. # 80775), and CD45RC (BD Biosciences, cat. # 554888, lot. # 23700). Tube #2 allowed for the quantification of the total proportions of recent thymic emigrants ($CD3^+$ $CD90^+$), late (mature) thymic emigrants ($CD3^+$ $CD90^-$ $CD45RC^+$), mature naive helper T-lymphocytes ($CD3^+CD45RC^+CD4^+$), mature memory helper T-lymphocytes ($CD3^+$ $CD45RC^-$ $CD4^+$), total B-lymphocytes ($CD45RC^+$), immature B-lymphocytes ($CD90^+$ $CD45RC^+$), and mature B-lymphocytes ($CD90^-$ $CD45RC^+$). In diabetes-prone BioBreeding rats, CD90 cells have a shorter lifespan and undergo increased apoptosis,

and thus there are fewer mature T-lymphocytes (Iwakoshi *et al.*, 1998). Therefore, we wanted to measure the proportions of recent and mature thymic emigrants to determine if zinc can elevate proportions of recent thymic emigrants improving diabetes associated immune dysfunction.

The procedure was as follows: The concentration of the cell suspension containing mesenteric lymph node cells and cell culture media was adjusted to 1×10^6 cells/mL.

Ex. $(7\times10^6$ cells/mL)(X) = $(1\times10^6$ cells/ml)(2.5 mL)

X = 0.357 mL cells + 2.143 mL cell culture media

One mL of cell suspension (1×10^6 cells/mL) was transferred into 12 × 15 mm round bottom staining tubes. Two million cells from each rat were used to create two tubes: combination 1 (CD3, CD4, CD8a, CD25) and combination 2 (CD3, CD4, CD90, CD45RC). The control tubes (unstained cells and isotypes: IgG1 FITC [BD Biosciences, cat. # 554679, lot. # 38941], IgG1 PE [BD Biosciences, cat. # 554680, lot. # 23699], IgM [BD Biosciences, cat. # 550883, lot. # 21211], IgG2 [BD Biosciences, cat. # 553458, lot. # 12618]) were made from the rat with the highest cell count. The cells were centrifuged for 8 minutes at 300 g at 4^{o}C, and the supernatant was rapidly decanted. The cell pellet was resuspended in 100 µL of label buffer (1 L of PBS, 10 mL charcoal dextran fetal bovine serum, and 1 g sodium azide [Fisher Scientific, cat. # S-227]). In the dark, antibodies were added to each tube. For the combination tubes, antibody cocktails were made according to the number of tubes being analyzed per day. Each combination tube then received 40 µL of the appropriate antibody cocktail. Volumes of each antibody that were used per tube were as follows: 1.25 µL CD3, 1.25 µL CD4, 0.5 µL CD8a, 0.3 µL

CD25, 1 µL CD90, 1.25 µL CD45RC, and 1.25 µL for all isotype controls. Tubes were incubated for 30 min in a covered ice box, to protect the flourochromes from light. After incubation, cells were washed with 2 mL of label buffer, then centrifuged at 300 g at 4^{o}C for 8 minutes and the supernatant was rapidly decanted. Cell pellets were resuspended in 1 mL 1% paraformaldehyde (made fresh each week) and stored at 4^{o}C until analysis on the flow cytometer (within 24 hours). Separate tubes containing one million unstained cells and each isotype were used for controls and ran on each day. Direct flow cytometry was performed using a BD LRS II flow cytometer (BD Biosciences, Mississauga, ON). The voltage was set at 596 for forward scatter, 349 for side-scatter, 514 for Alexa-Flour (FITC), 636 for PE, 544 for PEcy5, and 638 for APC. Compensation was set at 24.6% overlap for PE – FITC, 6.1% for PEcy5 – PE, 74.2% for PE – PEcy5, and 55% for APC – PEcy5. Laser excitation was tuned to 488 nm and 633 nm. The fluorescence signals were separated with the standard dichotic long-pass filters provided with the instrument and detected through 525 nm (FITC), 575 nm (PE), 675 nm (PEcy5), and 670 nm (APC) band-pass filters. Ten thousand events were collected and data was analyzed using BD FACSDiva™ software (Version 6.1.2). Refer to Appendix C-F for images and gating. Flow cytometry allowed for the quantification of cell proportions in the total sample.

Cytokine Secretion (TNF-α, IL-6, IFN-γ, IL-1β)

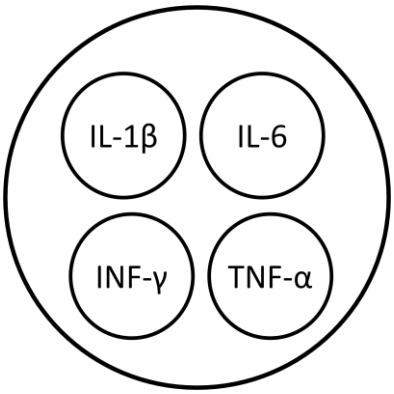
Many of the signs of type 2 diabetes are caused by chronic inflammation and an imbalance in cytokine secretion (Calle & Fernandez, 2012). In addition, zinc affects the levels of cytokines though altered gene expression in T-lymphocytes by altering the balance of Th1 and Th2 populations (John *et al.*, 2010). The first consequence of zinc deficiency is impaired immune function; levels of circulating cytokines (produced from

lymphocytes, granulocytes, and platelets) fluctuate in response to altered zinc status before a change in plasma zinc concentration can be detected (Foster & Samman, 2012; Prasad, 2008). Zinc deficiency may alter the balance of Th1 to Th2 cytokines leading to a pro-inflammatory state (Hosea, 2006).

Test Principle:

Refer to insulin assay for test principle.

Spot Layout:



Reagents:

1. Meso-Scale Discovery Custom Multiplex 4-Spot Mouse/Rat Cytokine Kit (cat. # 99906, Gaithersburg, MD)
2. Prepared cell culture media as follows:
    a. Mixed 2.6 g of RPMI-1640 with 200 mL ddH_20
    b. Added:

        2.5 mL 1M HEPES

        0.21 g sodium bicarbonate

        2.5 mL 100 mM sodium pyruvate

2.5 mL 100× non-essential amino acids

1 uL β-mercaptoethanol

c. Adjusted pH to 7. Topped up to 235 mL with ddH_20.

d. In cell culture hood added:

2.5 mL antibiotic/antimycotic

12.5 mL charcoal dextran fetal bovine serum

e. Sterile filtered, stored at 4^oC.

3. Prepared1 L of wash buffer (0.05% Tween-phosphate buffered saline [PBS-T]) (refer to insulin assay).
4. Prepared the cytokine calibrator combined working stock solution (1 µg/mL) by adding 10 µL of each 50 µg/mL individual cytokine stock (total of 40 µL) to 460 µL of cell culture media.
5. Prepared calibrator/standard curve which ranged from 0-40000 pg/mL.
    a. Prepared standard 1 by transferring 10 µL of the combined working stock solution to 240 µL of cell culture media.
    b. The remaining 6 controls were prepared by performing a 4-fold serial dilution using cell culture media (ex. added 50 µL standard 1 to 150 µL cell culture media to create standard 2). The last standard, standard 8 was a zero calibrator (cell culture media).
6. Prepared the detection antibody (refer to previous cytokine assay).
7. Prepared 2× read buffer (refer to previous cytokine assay).

Procedure:

Procedure followed the same methods as MCP-1. Samples were used undiluted. One sample was run on each plate as an internal control. Detection limits were 33.5-40000 pg/mL for IL-1β, 46.5-40000 pg/mL for IL-6, 41.4-40000 pg/mL IFN-γ, and 4.4-40000 pg/mL for TNF-α. Total serum cytokine content was calculated by adjusting for total blood volume [58 mL blood volume/kg body weight (Olfert, Cross, & McWillam, 1993)].

Western Blotting

The adipocyte plays a key role in inflammation since it secretes cytokines, such as TNF-α and MCP-1, which enhance inflammation and are produced through the activation of multiple signalling cascades involved in obesity and type 2 diabetes (Tataranni & Ortega, 2005). Jialal *et al.* (2012) found that circulating levels of pro-inflammatory cytokines and surface expression of TLRs (TLR-2, TLR-4) on monocytes are increased in patients with metabolic syndrome. TLRs are activated by lipids and induce pro-inflammatory cytokine production in macrophages and adipocytes (Calle & Fernandez, 2012; Suganami *et al.*, 2007). Therefore, obesity and type 2 diabetes may alter protein expression levels of inflammatory markers in adipocytes, enhancing inflammation.

GPR-39 is a zinc sensing receptor and a member of the ghrelin family that is activated by zinc, thereby inducing signal transduction pathways (Egerod *et al.*, 2011; Mlyniec, Budziszewska, Reczynski, Sowa-Kucma, & Nowak, 2013). GPR-39 is found in peripheral tissues with endocrine and metabolic functions; is involved in the control of appetite, the functioning of the gastrointestinal tract, energy production and metabolism

(Egerod *et al.*, 2011). GPR-39 deficiency has been associated with islet cell dysfunction, impaired insulin secretion in response to glucose, and glucose intolerance (Egerod *et al.*, 2011).

MCP-1 is a cytokine produced by inflammatory cells and its expression is enhanced in pro-inflammatory responses (Kolattukudy & Niu, 2012). Increased expression of MCP-1 in adipose tissue recruits macrophages and contributes to adipose tissue inflammation and insulin resistance (Kolattukudy & Niu, 2012). Increased circulating levels of MCP-1 are found in obesity, type 2 diabetes, and insulin resistance (Kolattukudy & Niu, 2012).

SOCS-3 can be rapidly induced by insulin, cytokines (especially TNF-α and IL-6), and fatty acids, reducing leptin sensitivity and inhibiting insulin signalling through a down-regulation of the JAK/STAT pathway (Emanuelli *et al.*, 2001; Munzberg & Myers, 2005; O'Connor, Sherry, Guest, & Freund, 2007; Palanivel *et al.*, 2012). Obesity elevates adipose tissue levels of SOCS-3 contributing to insulin resistance and glucose intolerance (Palanivel *et al.*, 2012). In *db/db* mice, SOCS-3 is elevated in adipose tissue, contributing to insulin resistance and reduced adiponectin production, via inhibition of the JAK/ STAT pathway (Kanatani *et al.*, 2007).

In *db/db* mice, obesity leads to elevated levels of TNF-α which induce SOCS-3 expression in insulin-sensitive tissues, especially adipose tissue (Emanuelli *et al.*, 2001; Kanatani *et al.*, 2007). Hotamasligil *et al.* (1993) found that obese adipose tissue secretes double the amount of TNF-α (expressed as the mass of TNF-α secreted per unit of tissue) as lean adipose tissue. Hotamasligil *et al.* (1993) also found that circulating TNF-α

concentrations were higher in obese *db/db* animals compared to lean. Long term exposure to TNF-α in adipocytes can lead to the down regulation of GLUT4, lowered fat and muscle glucose utilization, and peripheral insulin resistance (Hotamisligil, Shargill, & Spiegelman, 1993). Therefore, protein levels of GPR-39, MCP-1, SOCS-3, and TNF-α were measured in adipose tissue to determine the changes in expression associated with obesity, type 2 diabetes, and zinc status.

In addition, zinc has the ability to stimulate lipogenesis in adipocytes, in the absence of insulin (Coulston & Dandona, 1980). Zinc also helps to regulate the metabolism of adipose tissue by assisting in leptin secretion, the release of free fatty acids, and glucose uptake (Coulston & Dandona, 1980; Ott & Shay, 2001; Tang & Shay, 2001). Levels of zinc transporters (ZnT and ZIP) vary in lean versus obese humans, with higher levels in lean compared to obese individuals (Smidt *et al.*, 2007). ZnT2, ZnT3, ZnT6, and ZnT8 were expressed at higher levels in subcutaneous fat than visceral fat, and in fat tissue from lean versus obese individuals (Smidt *et al.*, 2007). Therefore, adipose tissue zinc transporter expression may be altered by obesity, disturbing zinc status, contributing to inflammation and exacerbating type 2 diabetes.

Antibodies for TLR-4 (Cell Signaling, cat. # 2219), TLR-2 (H-175) (Santa Cruz, cat. # sc-10739), ZIP7 (G-16) (Santa Cruz, cat. # sc-83858), ZnT6 (E-14) (Santa Cruz cat. # sc-161275), and ZIP2 (D-13) (Santa Cruz, cat. # sc-109874) were tested but didn't work.

Epididymal Fat Protein Extraction

Protein extraction from tissues is necessary for performing Western blotting (Ericsson & Nister, 2011).

Procedure:

To extract protein from epididymal fat, 200 mg of epididymal fat was weighed and placed in a mortar. The tissue was then covered with liquid nitrogen and ground to a fine powder using a pestle. Once the liquid nitrogen evaporated, 600 μL of 3× sample buffer (0.2 M Tris-HCl pH 6.8, 3% SDS, and 30% glycerol) was added to each sample and mixed well. Samples were allowed to lyse for 15 minutes in the mortar. After 15 minutes, samples were transferred to microcentrifuge tubes and sonicated (Sonic dismembrator, Model 100, Thermo Fisher Scientific Inc., Ottawa, ON) for 10 seconds. Samples were then centrifuged at 18000 g for 20 minutes. Protein lysates were aliquoted into microcentrifuge tubes and stored at -80^{o}C until analysis.

Protein Quantification

Test Principle:

Protein assays are used to determine the amount of protein in a sample. The protein assay was conducted using a Pierce bicinchoninic acid (BCA) kit (Thermo Scientific, Rockford, IL, cat. # 23225). The assay was a colourmetric assay using BCA to quantify the total protein content of a sample. In an alkaline medium, Cu^{+2} is reduced to Cu^{+1} by protein. Two molecules of BCA chelate with one Cu^{+1} to produce a purple colour. The amount of Cu^{+1} produced/purple colour is proportionate to the amount of

protein in the sample. The purple coloured chelated BCA-Cu^{+1} produced can be measured at an absorbance of 550 nm to give the amount of protein in the sample.

Reagents:

1. BCA™ Protein Assay Reagent A (Thermo Scientific, Rockford, IL, prod. # 23223): contains sodium carbonate, sodium bicarbonate, BCA™ detection reagent, and sodium tartrate in 0.1N sodium hydroxide
2. BCA™ Protein Assay Reagent B (Thermo Scientific, Rockford, IL, prod. # 23224)
3. Albumin Standard (Thermo Scientific, Rockford, IL, prod. # 23209): contains 2.0 mg/mL bovine serum albumin in 0.9% aqueous NaCl solution containing sodium azide

Procedure:

Step 1:

- Standard curve was prepared using 2 mg/mL bovine serum albumin (BSA) (cat. # 23209, Thermo Scientific, Rockford, IL, USA) and diluting with ddH_2O to create 0.2, 0.4, 0.6, 0.8, and 1 mg/mL BSA standards. DdH_2O was used as a blank.

Step 2:

- 10 µL of standard or samples was added into each well of a 96-well plate (cat. # 167008, NUNC™, Roskilde Denmark) in triplicate.

Step 3:

- Reaction reagent mixture was mixed by adding the appropriate amount (1:50) of BCA Reagent B to BCA Reagent A.
- 200 µL of reaction mixture was added to each well.
- The plate was covered with the plate lid and sealed using parafilm.
- Samples were incubated at 37^{o}C for 30 minutes.

Step 4:

- Plate was removed from incubator; bubbles were popped by gently blowing on the plate.
- Plate was read on the FLUOstar Omega (BMG LABTECH, Ortenberg/Germany) plate reader at an absorbance of 550 nm.

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Test Principle:

SDS-PAGE is a technique used to separate proteins based on their molecular weights. Protein lysates are heated and then loaded into a polyacrylamide gel. An electrical current is used to pull the negatively charged proteins through the gel. The molecular weight of the protein determines how fast it is pulled through the gel; smaller, lower molecular weight proteins travel faster and farther than large molecular weight proteins. Once the proteins are separated by their molecular weights, the proteins can be transferred to a polyvinylidene fluoride (PVDF) membrane to be used for Western blotting.

Reagents:

Table 4. Western Blotting Reagents

% Acrylamide Gel	**20% Acrylamide (mL)**	**0.5 M Tris-HCl pH 8.8 (mL)**	**ddH_2O (mL)**	**10% APS (µL)**	**10% SDS (µL)**	**TEMED (µL)**
10% Separating Gel	10	4.5	5.3	200	200	16
15% Separating Gel	15	4.5	0.3	200	200	16
% Acrylamide Gel	**20% Acrylamide (mL)**	**0.5 M Tris-HCl pH 6.8 (mL)**	**ddH_2O (mL)**	**10% APS (µL)**	**10% SDS (µL)**	**TEMED (µL)**
5% Stacking Gel	2	2	3.76	80	80	20

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

Other reagents

- t-amyl alcohol
- 10% bromophenol blue (BPB) (w/v)
- 2-mercaptoethanol (β-ME) (14.16 M)
- 5× running buffer: 200 mL 10% SDS, 288 g glycine, 60.6 g Tris-base, top up to 4 L with ddH_2O

- 5× transfer buffer: 75.75 g Tris-base, 242.5 g glycine, top up to 4 L with ddH_2O
- Methanol
- 5× Tris-buffered saline with Tween-20 (TBST): 600 mL 5 M NaCl, 40 mL 1 M Tris-HCl pH 7.4, 10 mL Tween-20, ddH_2O to yield a final volume of 4 L

Procedure:

To prepare separating gel, 2 glass plates and 2 spacers were cleaned with ddH_2O. The gasket was wrapped around the bottom of one of the glass plates and the plates were sandwiched together with casting clips. The separating gel was prepared (Refer to Table 4) and pipetted into the space between the two glass plates. T-amyl alcohol was pipetted onto the top of the gel. A 50-well comb was added and more t-amyl alcohol was added to the top of the gel. The gel was allowed to polymerize for 45 minutes. After polymerization, the t-amyl alcohol was rinsed off with ddH_2O and allowed to dry slightly. Next, the stacking gel was prepared (refer to Table 4) and pipetted down the side of the comb so that it filled all the wells. The stacking gel was allowed to polymerize for 10 minutes.

While the stacking gel polyermized, the protein samples were prepared by aliquoting 20 μg of protein into a microcentrifuge tube (see below for sample protein calculation). A1:1 ratio of 10% BPB and β-ME was prepared and added to each protein sample so that each sample contained 10% dye (ex. 20 μL protein would have 2 μL of dye). Samples were then placed in a water bath and microwaved on high for 3 minutes to denature the proteins.

Volume of protein sample calculation:

$$\frac{\text{20 μg protein to load onto gel}}{\text{Protein concentration from protein assay (μg/mL)}} = \text{Volume of protein sample needed (μL)}$$

Once the stacking gel polymerized, the casting clamps were removed and the gasket was peeled off. Half of the working running buffer solution (200 mL 5× running buffer and 800 mL ddH_2O) was poured into the bottom of the electrophoresis tank and the plates were gently placed into the tank, while ensuring no bubbles were created under the gel. The plates were clamped to the electrophoresis apparatus and the comb was removed from the wells. The rest of the running buffer was poured into the middle of the tank until each well was filled with buffer. A protein molecular weight marker was loaded into the first and last 2 wells of every gel using a glass syringe. Samples were then loaded into each well. Once samples were loaded, the electrophoresis tank was moved into the cold room (4^{o}C), connected to a power supply, and ran at 70 mA for about 1.5 hours or until the dye front reached the bottom of the gel.

After the gel was done running, the gel was removed from the tank and the glass plates were pried apart. The stacking gel was removed from the separating gel and discarded. The gel was then turned onto a piece of blotting paper. A PVDF membrane was labelled and dipped into methanol for 1 minute before being placed on top of the gel. Another piece of blotting paper was placed on top of the PVDF membrane and then this sandwich was placed between two pieces of fibre board. The sandwich was placed into a container filled with the working transfer buffer solution (500 mL methanol, 400 mL 5× transfer buffer, topped up to 3 L with ddH_2O). Bubbles were pushed out using a roller and the sandwich was placed into the transfer gel cassette. The cassette was placed into

the transfer tank, filled with working transfer buffer, and moved to the cold room (4^{o}C). The gel was allowed to transfer on a stir plate for 45 minutes at 100 V. Once the transfer was complete, everything but the PVDF membrane was discarded and the PVDF membrane was placed into a container filled with 1× TBST for storage until Western blotting.

Immunoblotting

Test Principle:

Western blotting is a technique used to quantify the amount of specific proteins in a sample or tissue (Towbin, Staehelin, & Gordon, 1979). A primary antibody is applied. Then a secondary antibody conjugated to horseradish peroxidase (HRP) is applied and binds to the primary antibody. A chemiluminescent substrate is added and causes the HRP to catalyze a reaction that results in the emission of light. The light produced results in a visible black band on audioradiographic film. The intensities of specific bands are measured using a densitometer which measures transmitted light through a photographic negative and compares it to a known incident light, and computes the ratio. The amount of light produced is directly proportional to the amount of protein present in the sample.

Reagents and Materials:

- 3% BSA in TBST
- 5× TBST: 600 mL 5 M NaCl, 40 mL 1 M Tris-HCl pH 7.4, 10 mL Tween-20, ddH_2O to yield a final volume of 4 L
- 1% BSA-TBST
- Primary antibody

- Secondary antibody conjugated with HRP
- Luminata™ Crescendo Western HRP substrate (EMD Millipore Corporation, Billerica, MA, USA, cat. # WBLUR0100)
- Autoradiographic film (CL-XPosure™ Clear Blue X-Ray Film, Thermo Scientific, Rockford, IL, cat. # PI-34097)

Procedure:

Membranes were placed in a blotting box and blocked with 40 mL of 3% BSA-TBST for 1 hour at room temperature on a shaker. After 1 hour, the 3% BSA-TBST was poured off and the primary antibody was added at the appropriate concentration (refer to Table 5) diluted in 40 mL of 3% BSA-TBST. The primary antibodies were placed on the shaker for 1 hour at room temperature or overnight at 4^{o}C (refer to Table 5). Next, the primary antibody was poured back into a 50 mL conical tube and frozen at -20^{o}C for future use. Membranes were washed 4 times for 5 minutes each with 1× TBST. Secondary antibodies were then added at appropriate concentrations (refer to Table 5) diluted in 40 mL of 1% BSA-TBST. The secondary antibody was applied for 1 hour at room temperature on a shaker. After 1 hour, the membranes were washed again with 1× TBST (4 washes, 5 minutes each). Membranes were then dipped into Luminata™ Crescendo Western HRP electrochemiluminescent substrate and then placed between two acetate sheets and placed into an autoradiography cassette. In the dark, blue X-ray film was exposed to the membranes for an appropriate amount of time (refer to Table 5), until the band of interest was visible.

A densitometer (trace analysis with a GS 800 Imaging Densitometer, Bio-Rad Laboratories, Hercules, CA) and Quantity One software (Version 4.5.0, Bio-Rad Laboratories, Hercules, CA) were used to determine the intensity of the bands. Due to potential differences in protein loading across lanes, the intensity (trace quantity) of the protein band of interest was divided by the intensity of the loading control (β-Actin).

Table 5. Western Blotting Antibodies and Conditions

Primary Ab	1^o Ab Concentration & Incubation Time	2^o Ab	2^o Ab Concentration & Incubation Time	Molecular Weight (kDa)	Exposure Time (seconds)	Company	Catalogue Number
B-Actin	1:1000, 1 hour	Rabbit HRP	1:10 000, 1 hour	45	2	Cell Signaling	4967L
GPR-39	1:500, O/N	Rabbit HRP	1: 10 000, 1 hour	51	10	Abcam	ab39227
MCP-1	1:2000, 1 hour	Rabbit HRP	1:15 000, 1 hour	25	10	Abcam	ab25124
SOCS-3	1:1000, 1 hour	Rabbit HRP	1:10 000, 1 hour	26	120	Cell Signaling	2923A
TNF-α (L-19)	1:2000, 1 hour	Goat HRP	1:10 000, 1 hour	26 transmembrane (precursor), 17 soluble (mature)	180	Santa Cruz	sc-1351
ZnT3 (G-14)	1:500, O/N	Goat HRP	1:5000, 1 hour	48	8	Santa Cruz	sc-27508

Ab = antibody, 1^o = primary, 2^o = secondary, O/N = overnight, HRP = horseradish peroxidase

Statistical Analyses

SAS statistical software (SAS software release 9.2, SAS Institute, Cary, NC, USA) was used for statistical analyses. Repeated measures analysis of variance (ANOVA) was used for time course data, for example, weekly body weights or glucose concentrations during OGTT, and one way ANOVA was used for endpoint data. ANOVA was followed by means testing with Duncan's multiple range test if there were significant main effects. Non-normal and non-homogeneous data were analyzed following log transformation, or non-parametric testing was used. Non-parametric testing is used to analyze data that does not have a set distribution or fixed structure. For non-parametric testing, the Kruskal-Wallis test was followed by least significant difference post hoc testing with Tukey correction for multiple comparisons. Pearson (normal data) or Spearman's (non-normal data) correlations were used to determine the relationship between two sets of data. Outliers (± 2.5 standard deviations from the mean) were removed from the data set. Data are reported as means ± the standard error of the mean. Differences were considered significant at $P < 0.05$.

V. RESULTS

Diabetes Parameters

Feed Intake and Body Weight

ZDF rats consumed an average of 31% (26-35%) more feed per day than lean rats (Table 6). ZDF rats gained 40% more weight over the 8 week study than lean ZDF rats. ZDF rats weighed more than lean ZDF rats throughout the 8 weeks of the study (Figure 2) and at the end of the study (Table 6). There was no effect of dietary zinc on feed intake or body weight in ZDF rats.

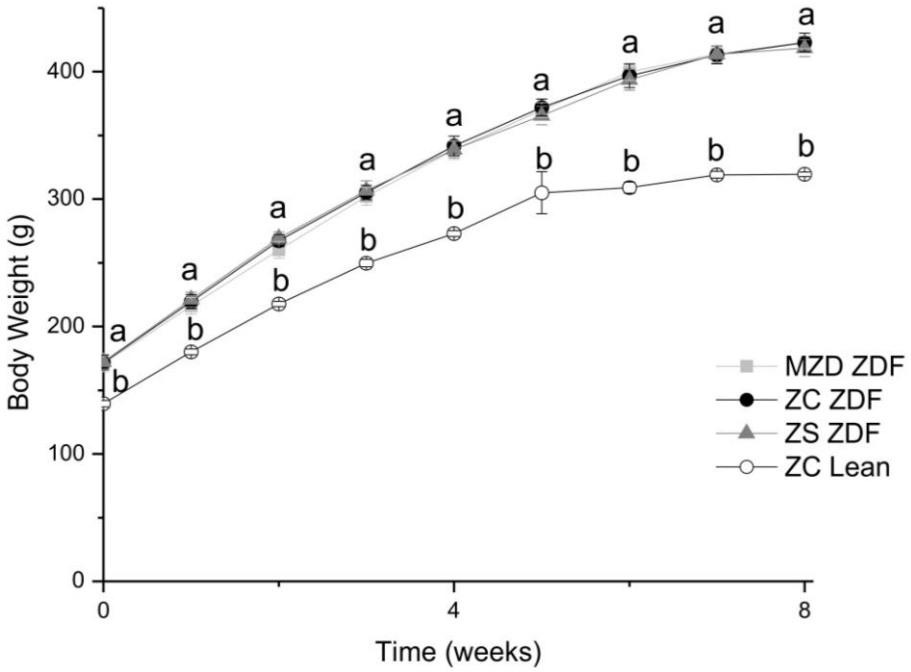


Figure 2. Weekly Body Weights. Data expressed as means ± SEM, n = 10 except for ZC Lean and ZS where n = 7 and 9, respectively. There were significant main effects of time and time × diet interaction. Different letters indicate significant differences ($P < 0.05$) among means at the same time point. MZD ZDF = ZDF fed marginal zinc deficient diet, ZC ZDF = ZDF fed zinc control diet, ZS ZDF = ZDF fed zinc supplemented diet, ZC Lean = lean ZDF (+/?) fed zinc control diet.

Table 6. Feed Intake and Final Body Weight

	ZDF			
	MZD	**ZC**	**ZS**	**ZC Lean**
Feed Intake (g/day)[1]	26.6 ± 1.4[a]	28.4 ± 1.8[a]	27.7 ± 2.2[a]	21.1 ± 1.4[b]
Weight Gain (g)[1]	252 ± 5[a]	251 ± 5[a]	246 ± 5[a]	180 ± 4[b]
Final Body Weight (g)[1]	423 ± 5[a]	423 ± 7[a]	419 ± 7[a]	319 ± 2[b]

Values are means ± standard error of the mean (SEM), n = 10 except for ZC Lean and ZS where n = 7 and 9, respectively. Different superscript letters indicate significant differences ($P < 0.05$) among means. MZD = ZDF fed marginal zinc deficient diet, ZC = ZDF fed zinc control diet, ZS = ZDF fed zinc supplemented diet, ZC Lean = lean ZDF (+/?) fed zinc control diet.

[1]Data were log transformed prior to ANOVA.

Body Composition

At 8 weeks, ZDF rats had higher body weight due to greater fat mass. Body composition analyses revealed that ZDF rats had 6-fold higher body fat and 13% less lean body tissue as well as lower total body water compared to lean rats (Table 7). There was no effect of dietary zinc on body composition in ZDF rats.

Table 7. *In Vivo* Body Composition at 8 Weeks[1]

	ZDF			
	MZD	**ZC**	**ZS**	**ZC Lean**
Fat Mass (g)[2]	164 ± 2[a]	170 ± 3[a]	169 ± 3[a]	27.9 ± 1.6[b]
Lean Mass (g)	247 ± 6[a]	239 ± 7[a]	242 ± 4[a]	280 ± 5[b]
Free Water (g)	1.36 ± 0.21	1.31 ± 0.08	1.09 ± 0.17	1.00 ± 0.08
Total Water (g)	209 ± 5[a]	202 ± 6[a]	205 ± 3[a]	235 ± 5[b]

Values are means ± SEM, n = 10 except for ZC Lean and ZS where n = 7 and 9, respectively. Different superscript letters indicate significant differences ($P < 0.05$) among means. MZD = ZDF fed marginal zinc deficient diet, ZC = ZDF fed zinc control diet, ZS = ZDF fed zinc supplemented diet, ZC Lean = lean ZDF (+/?) fed zinc control diet.

[1]*In vivo* body composition obtained by EchoMRI-700™ whole body QMR instrument (Echo Medical Systems, Houston, TX).

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

Obesity Measurements

ZDF rats had about 2 cm shorter tail lengths than lean rats despite no differences in body length (Table 3). MZD and ZS reduced Lee's index, a measure of adiposity (Cox, Laughton, & Powley, 1985), compared to ZC in ZDF rats (Table 8). Lean rats had reduced Lee's index versus ZDF rats. There was a positive correlation between Lee's index and body fat mass ($r = 0.61$, $P < 0.0001$) (Figure 3) and there was a positive correlation between body mass index (BMI) and body fat mass ($r = 0.73$, $P < 0.0001$). There was a negative correlation between Lee's index and total body water ($r = -0.52$, $P = 0.001$) and there was a trend for a weak correlation between BMI and total body water ($r = -0.31$, $P = 0.06$). ZDF rats had a greater BMI than lean rats, and ZS ZDF had a reduced BMI compared to ZC ZDF (Table 8).

ZDF rats had greater epididymal, mesenteric, and peri-renal fat pad mass compared to lean rats but absolute fat pad mass did not differ among dietary zinc groups in ZDF rats (Table 8). After adjusting for body weight, MZD reduced peri-renal fat mass by 5% and 9%, compared to ZC ZDF and ZS ZDF rats, respectively (Table 8). Therefore, MZD resulted in 7% lower total visceral fat mass than ZS in ZDF rats (Figure 4).

Table 8. Obesity Measurements

	ZDF			
	MZD	**ZC**	**ZS**	**ZC Lean**
Body Length (cm)	21.3 ± 0.1	21.0 ± 0.2	21.2 ± 0.1	21.6 ± 0.2
Tail Length (cm)[2]	17.1 ± 0.1[a]	17.0 ± 0.2[a]	17.0 ± 0.2[a]	19.0 ± 0.1[b]
Lee's Index ($g^{0.33}$/mm)[2]	0.0345 ± 0.0001[b]	0.0350 ± 0.0001[a]	0.0345 ± 0.0001[b]	0.0310 ± 0.0002[c]
BMI (g/cm^2)[2]	0. 932 ± 0.001[ab]	0.956 ± 0.001[a]	0.928 ± 0.001[b]	0.684 ± 0.001[c]
Epididymal Fat (g)[1]	9.13 ± 0.22[a]	9.19 ± 0.16[a]	9.24 ± 0.16[a]	2.84 ± 0.16[b]
(g/100 g BW)[2]	2.16 ± 0.06[a]	2.18 ± 0.05[a]	2.21 ± 0.05[a]	0.89 ± 0.05[b]
Peri-renal Fat (g)[2]	14.7 ± 0.3[a]	15.4 ± 0.4[a]	15.9 ± 0.3[a]	4.17 ± 0.2[b]
(g/100 g BW)[1]	3.46 ± 0.05[a]	3.64 ± 0.07[b]	3.80 ± 0.06[b]	1.30 ± 0.05[c]
Mesenteric Fat (g)[2]	5.59 ± 0.19[a]	5.74 ± 0.28[a]	5.94 ± 0.29[a]	1.78 ± 0.09[b]
(g/100 g BW)[2]	1.32 ± 0.04[a]	1.35 ± 0.06[a]	1.42 ± 0.06[a]	0.56 ± 0.03[b]

Values are means ± SEM, n = 10 except for ZC Lean and ZS ZDF where n = 7 and 9, respectively. Different superscript letters indicate significant differences ($P < 0.05$) among means. MZD = ZDF fed marginal zinc deficient diet, ZC = ZDF fed zinc control diet, ZS = ZDF fed zinc supplemented diet, ZC Lean = lean ZDF (+/?) fed zinc control diet. BMI = body mass index, BW = body weight.

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

[2]Data were log transformed prior to ANOVA.

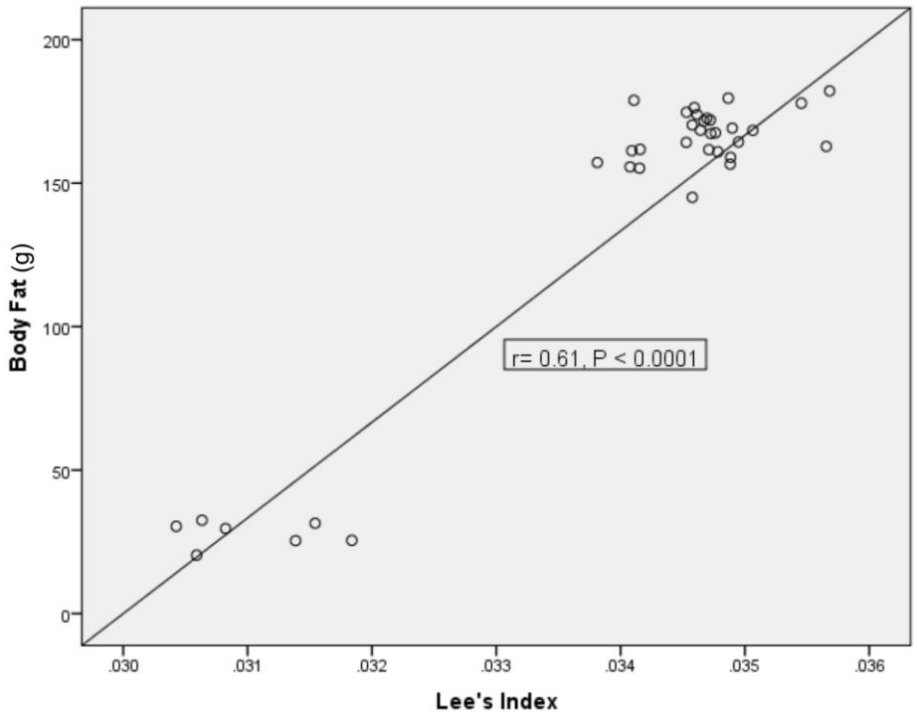


Figure 3. Correlation between body fat mass and Lee's index. n = 36.

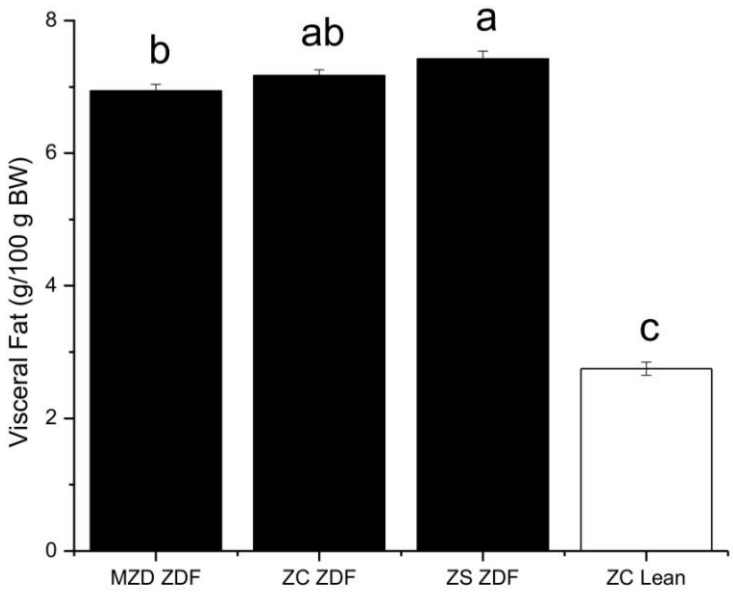


Figure 4. Visceral fat mass. Visceral fat mass is the sum of epididymal, peri-renal and mesenteric adipose tissues. Data expressed as means ± SEM, n = 10 except for ZC Lean and ZS ZDF where n = 7 and 9, respectively. Different letters indicate significant differences ($P < 0.05$). MZD = ZDF fed marginal zinc deficient diet, ZC = ZDF fed zinc control diet, ZS = ZDF fed zinc supplemented diet, ZC Lean = lean ZDF (+/?) fed zinc control diet. Non-parametric analysis was used.

GPR-39

GPR-39 is a member of the ghrelin receptor family which is found in peripheral tissues, endocrine and metabolic tissues (Egerod *et al.*, 2011). GPR-39 is a zinc sensing receptor which is activated by zinc, thereby activating signal transduction pathways involved in energy metabolism (Egerod *et al.*, 2011; Mlyniec, Budziszewska, Reczynski, Sowa-Kucma, & Nowak, 2013). Protein levels of GPR-39 in epididymal fat were not different between ZDF and lean rats (Table 9, Figure 5).

Table 9. Epididymal Fat Protein Levels of GPR-39

	ZDF			
	MZD	**ZC**	**ZS**	**ZC Lean**
GPR-39	2.20 ± 0.32	1.76 ± 0.28	1.96 ± 0.36	2.68 ± 0.32

Values are means ± SEM, n = 10 except for ZC Lean and ZS where n = 7 and 9, respectively. GPR-39 was analyzed by Western blotting. Relative intensities of the bands were quantified by a densitometer. All data were adjusted by the loading control (β-actin) to control for differences in protein loading. Data expressed in arbitrary units. Absence of letters indicates no significant differences (P > 0.05) among means. MZD = ZDF fed marginal zinc deficient diet, ZC = ZDF fed zinc control diet, ZS = ZDF fed zinc supplemented diet, ZC Lean = lean ZDF (+/?) fed zinc control diet.

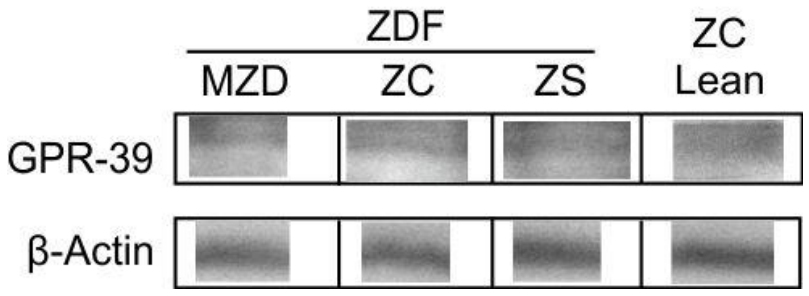


Figure 5. Representative blots of GPR-39. Figure was obtained from Western Blot analyses.

Glucose Tolerance and Insulin Resistance

Fasting Serum Glucose and Insulin

After a 5 hour fast in metabolic cages, fasting serum glucose was not different between ZDF and lean rats over the 8 weeks of the study (Figure 6A). ZDF rats had higher fasting serum insulin concentrations at 0, 4 and 8 weeks compared to lean rats (Figure 6B). ZDF rats had higher HOMA-IR scores, a measure of insulin resistance, throughout the 8 week study compared to lean rats (Figure 7).

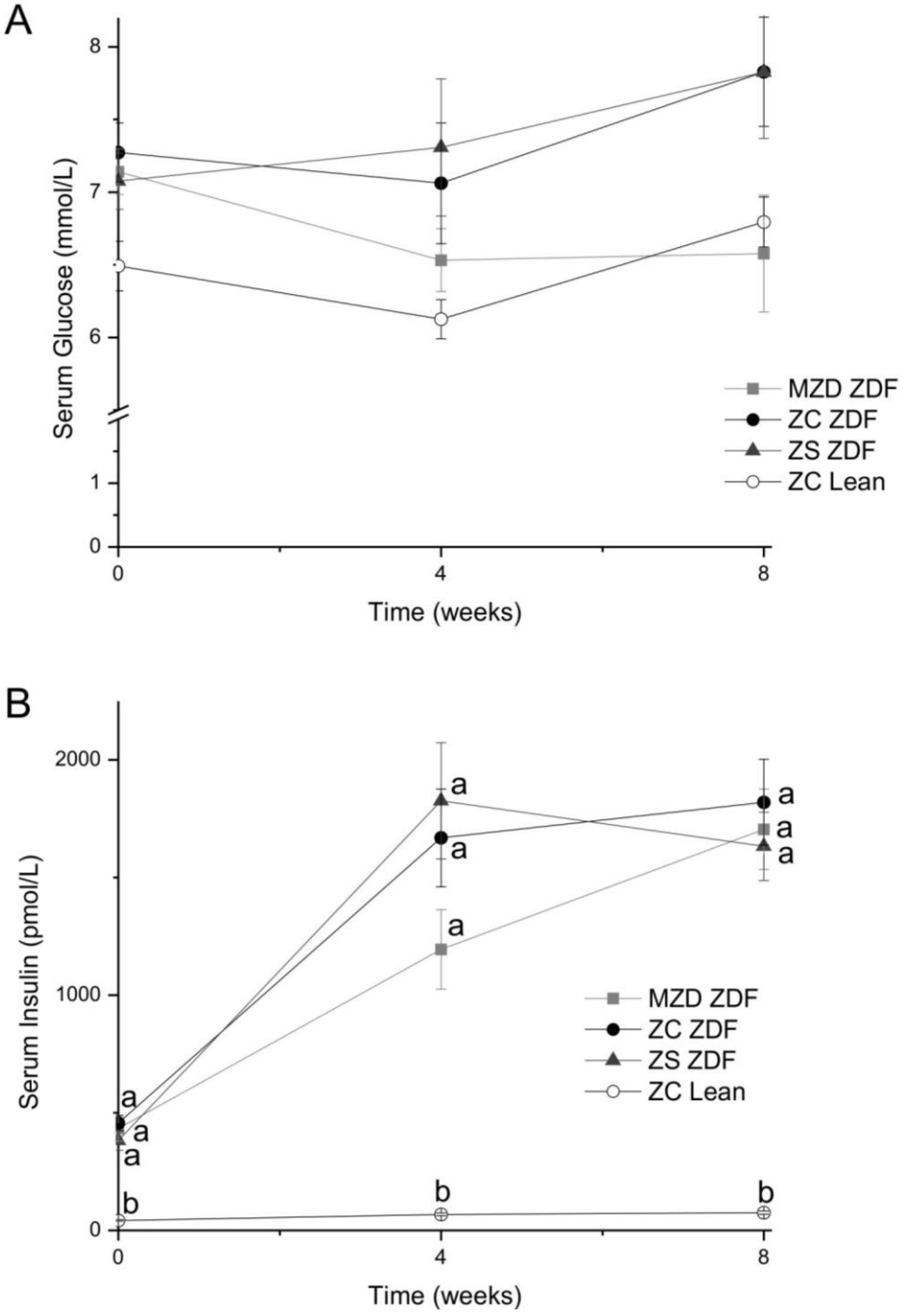


Figure 6. After a 5 hour fast, serum glucose (A) and insulin (B) at weeks 0, 4, and 8. Data expressed as means ± SEM, n = 10 except for serum insulin MZD = 9 at week 0, and ZC Lean and ZS where n = 7 and 9, respectively. There were significant main effects of time and time × diet interaction for insulin. Different letters indicate significant differences ($P < 0.05$) among means at the same time point. MZD = ZDF fed marginal zinc deficient diet, ZC = ZDF fed zinc control diet, ZS = ZDF fed zinc supplemented diet, ZC Lean = lean ZDF (+/?) fed zinc control diet.

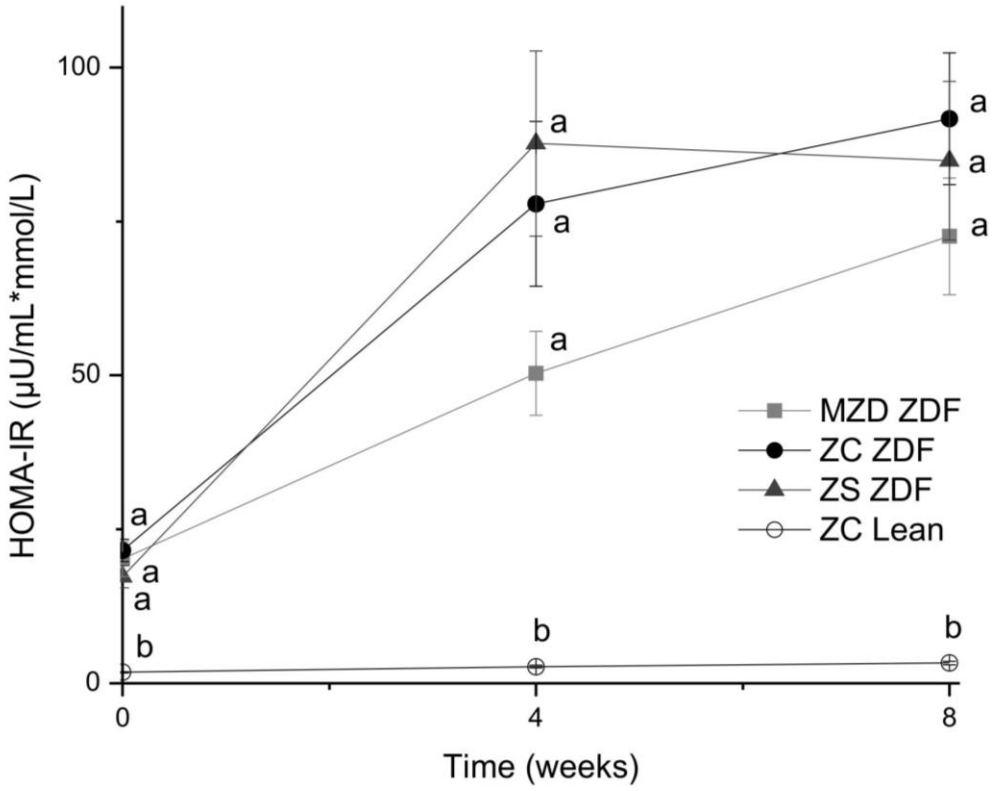


Figure 7. HOMA-IR at weeks 0, 4, and 8. Data expressed as means ± SEM, n = 10 except for ZC Lean and ZS where n = 7 and 9, respectively. There were significant main effects of time and time × diet interaction. Different letters indicate significant differences ($P < 0.05$) among means at the same time point. MZD = ZDF fed marginal zinc deficient diet, ZC = ZDF fed zinc control diet, ZS = ZDF fed zinc supplemented diet, ZC Lean = lean ZDF (+/?) fed zinc control diet.

Oral Glucose Tolerance Testing

There was no difference in absolute serum glucose concentrations among dietary zinc groups throughout the OGTT (Appendix A). After adjusting for baseline serum glucose values, MZD had elevated blood glucose concentrations at 60 minutes of the OGTT compared to all other groups although it was not statistically significant (Figure 8A). Insulin concentrations were higher in ZDF rats compared to lean rats, but there was no effect of zinc during the OGTT (Appendix B). After adjusting for baseline insulin concentrations, there was no difference in insulin concentrations during OGTT between ZDF and lean rats (Figure 8B). ZDF rats had a higher area-under-the-curve (AUC) for insulin (Figure 9A) but the AUC for glucose was not different between ZDF and lean rats (Table 10). Therefore, the AUC ratio for glucose to insulin (Figure 9B) was lower and the glucose to insulin index (Table 10) was higher in ZDF rats compared to lean rats during OGTT. The ISI (calculated from fasting glucose and insulin, and the AUC for glucose and insulin during OGTT) was 95% lower in ZDF rats compared to lean rats (Table 10).

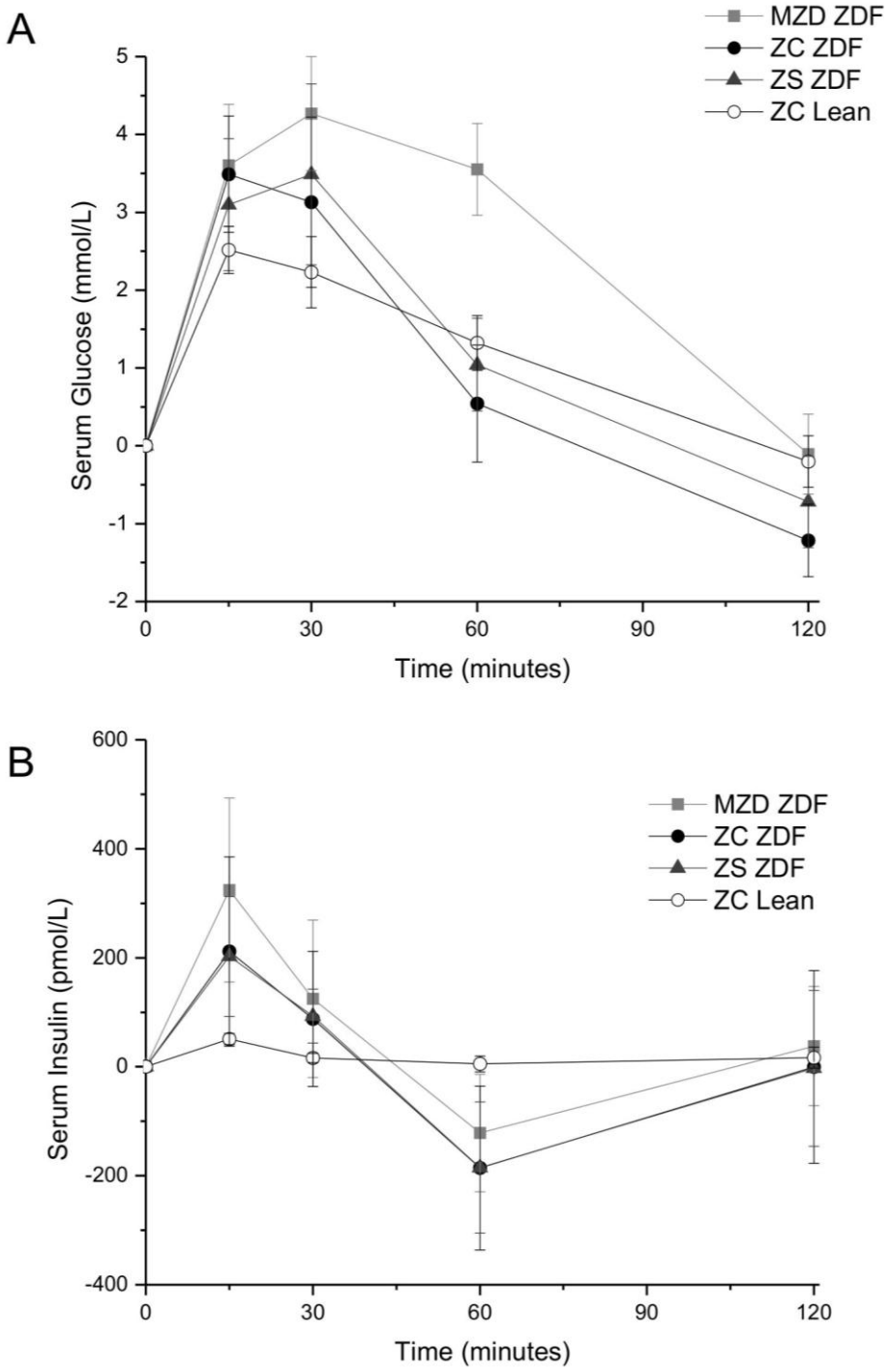


Figure 8. Serum glucose (A) and insulin (B) calculated as change from baseline during OGTT. Data expressed as means ± SEM, n = 10 except for ZC Lean and ZS where n = 7 and 9, respectively. There were significant main effects of time but not time × diet interaction for glucose and insulin. Absence of letters indicates no significant differences ($P > 0.05$) among means at the same time point.

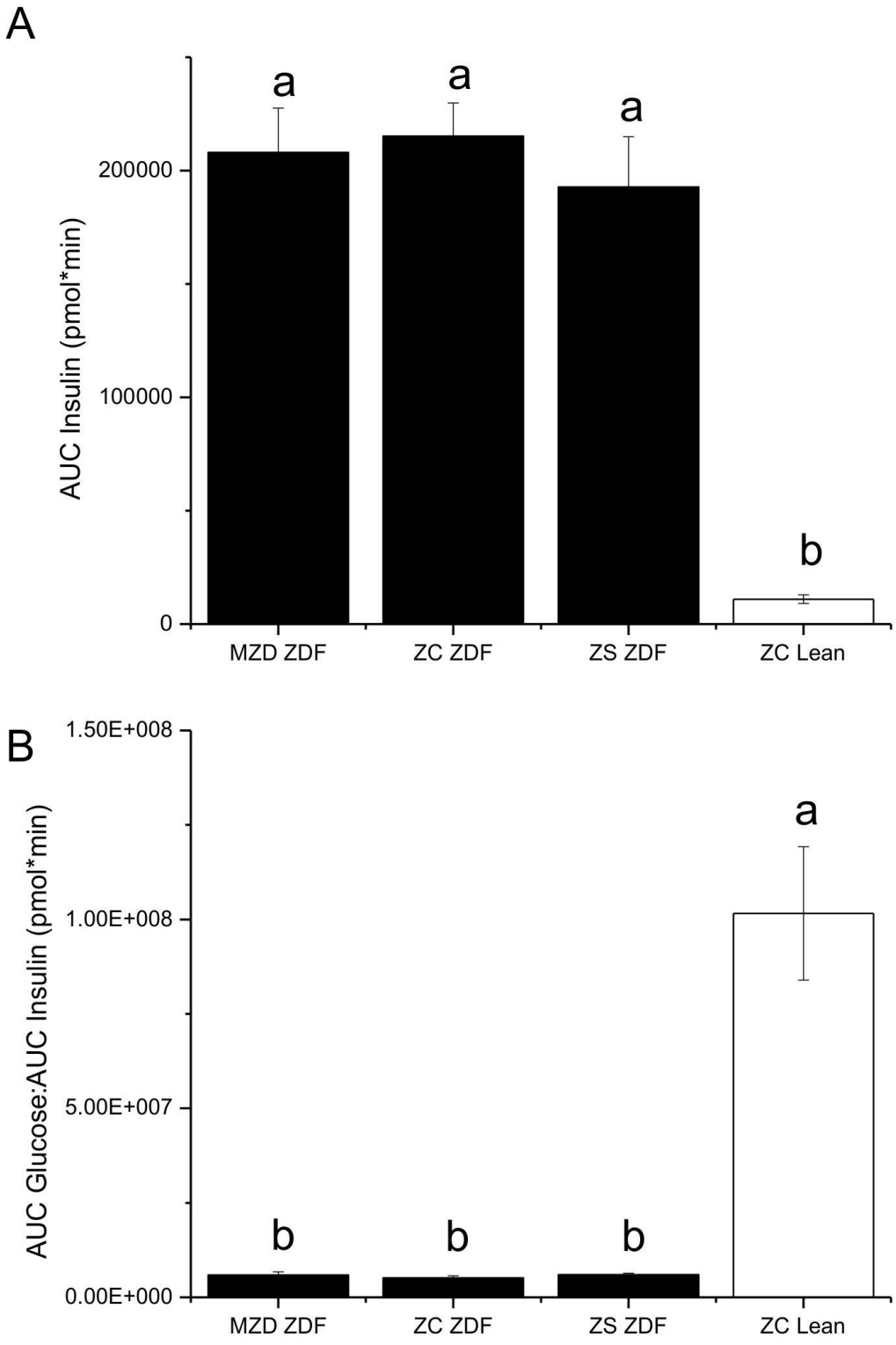


Figure 9. AUC insulin (A) and AUC glucose to insulin ratio (B). Data expressed as means ± SEM, n = 10 except for ZC Lean and ZS where n = 7 and 9, respectively. Different letters indicate significant differences ($P < 0.05$) among means. Data were log transformed prior to ANOVA.

Table 10. AUC for Glucose, Glucose-Insulin Index, and ISI

	ZDF			
	MZD	**ZC**	**ZS**	**ZC Lean**
AUC Glucose (mmol/L*min)	$1.99E^{13} \pm 7.92E^{11}$	$1.75E^{13} \pm 9.80E^{11}$	$1.96E^{13} \pm 1.53E^{11}$	$1.72E^{13} \pm 6.38E^{11}$
Glucose-Insulin Index (pmol*mmol*min^2)[1]	$2.30\ E^{8} \pm 1.90\ E^{7\ a}$	$2.20\ E^{8} \pm 2.10\ E^{7\ a}$	$2.20\ E^{8} \pm 4.00\ E^{7\ a}$	$1.00\ E^{7} \pm 1.76\ E^{6\ b}$
ISI[1, 2]	$2.20\ E^{-7} \pm 2.00\ E^{-8\ b}$	$1.90\ E^{-7} \pm 2.00\ E^{-8\ b}$	$2.20\ E^{-7} \pm 3.00\ E^{-8\ b}$	$4.70\ E^{-6} \pm 4.20\ E^{-7\ a}$

Values are means ± SEM, n = 10 except for ZC Lean and ZS where n = 7 and 9, respectively. Different superscript letters indicate significant differences ($P < 0.05$) among means. MZD = ZDF fed marginal zinc deficient diet, ZC = ZDF fed zinc control diet, ZS = ZDF fed zinc supplemented diet, ZC Lean = lean ZDF (+/?) fed zinc control diet. ISI (Paulsen, Vrang, Larsen, Larsen, & Jelsing, 2010) calculation is as follows:

$$ISI = 10000 \div \sqrt{[(FPG \times FPI) \times (AUG\ OGTT\ Glucose \times AUC\ OGTT\ Insulin)]}$$

FPG = fasting plasma glucose, FPI = fasting plasma insulin, AUC = area under the curve.
[1]Data were log transformed prior to ANOVA.
[2]Myllynen *et al.* (1987).

Termination Serum Glucose and Insulin

After a 12 hour (overnight) fast, serum glucose concentrations were not different between ZDF and lean rats (Figure 10A). ZDF rats had substantially higher (21-fold) insulin concentrations compared to lean rats (Figure 10B). ZDF rats had 23-fold higher HOMA-IR scores, a measure of peripheral insulin resistance (Figure 11A), and 17-fold higher HOMA-BCF scores, a measure of beta-cell function (Figure 11B), compared to lean rats. There was no effect of dietary zinc on serum glucose or insulin, or HOMA-IR or HOMA-BCF, after a 12 hour fast in ZDF rats.

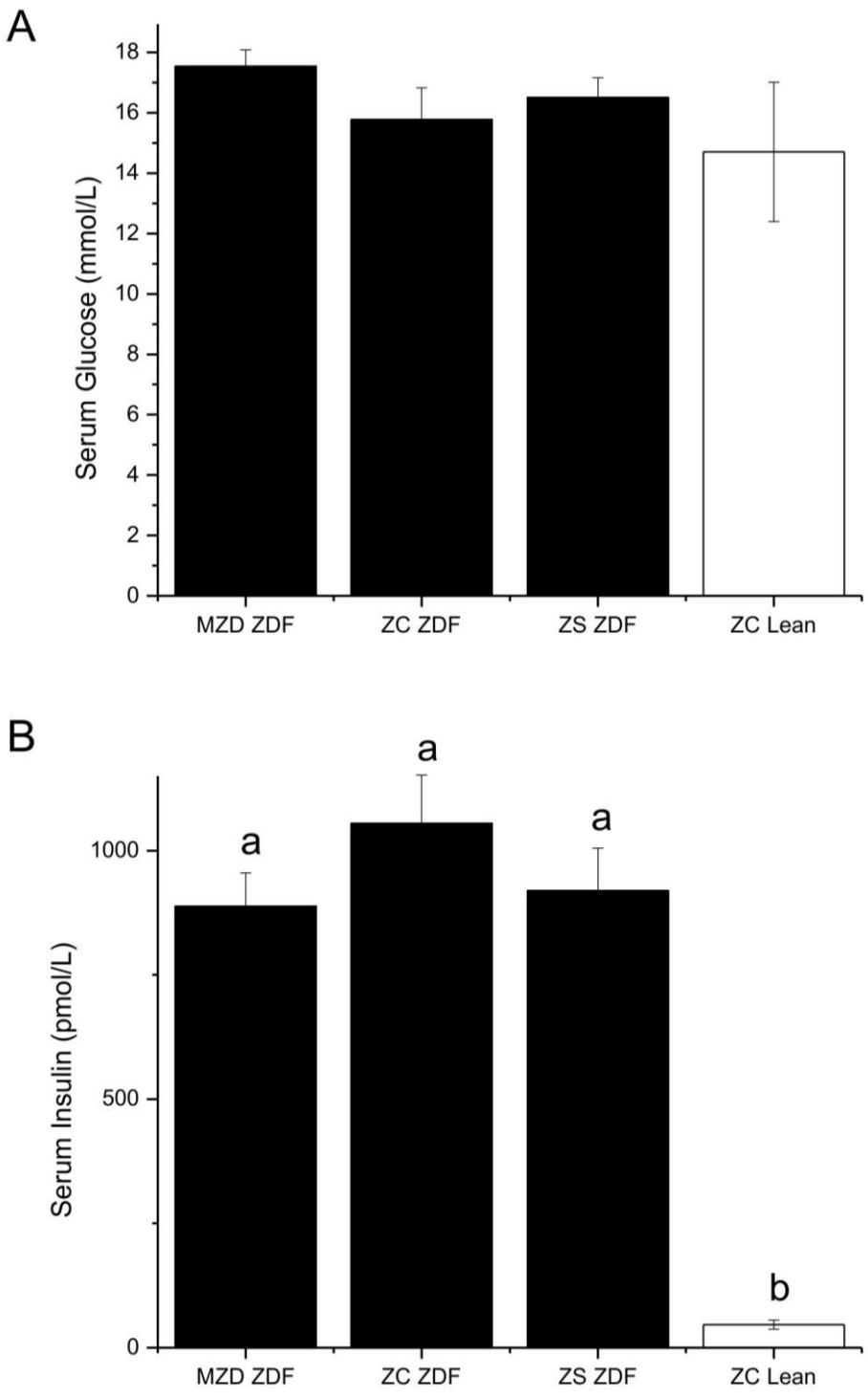


Figure 10. After a 12 hour fast, serum glucose (A) and insulin (B) concentrations at week 8. Data expressed as means ± SEM, n = 10 except for ZC Lean and ZS where n = 7 and 9, respectively. Different letters indicate significant differences ($P < 0.05$) among means. Insulin data were log transformed prior to ANOVA.

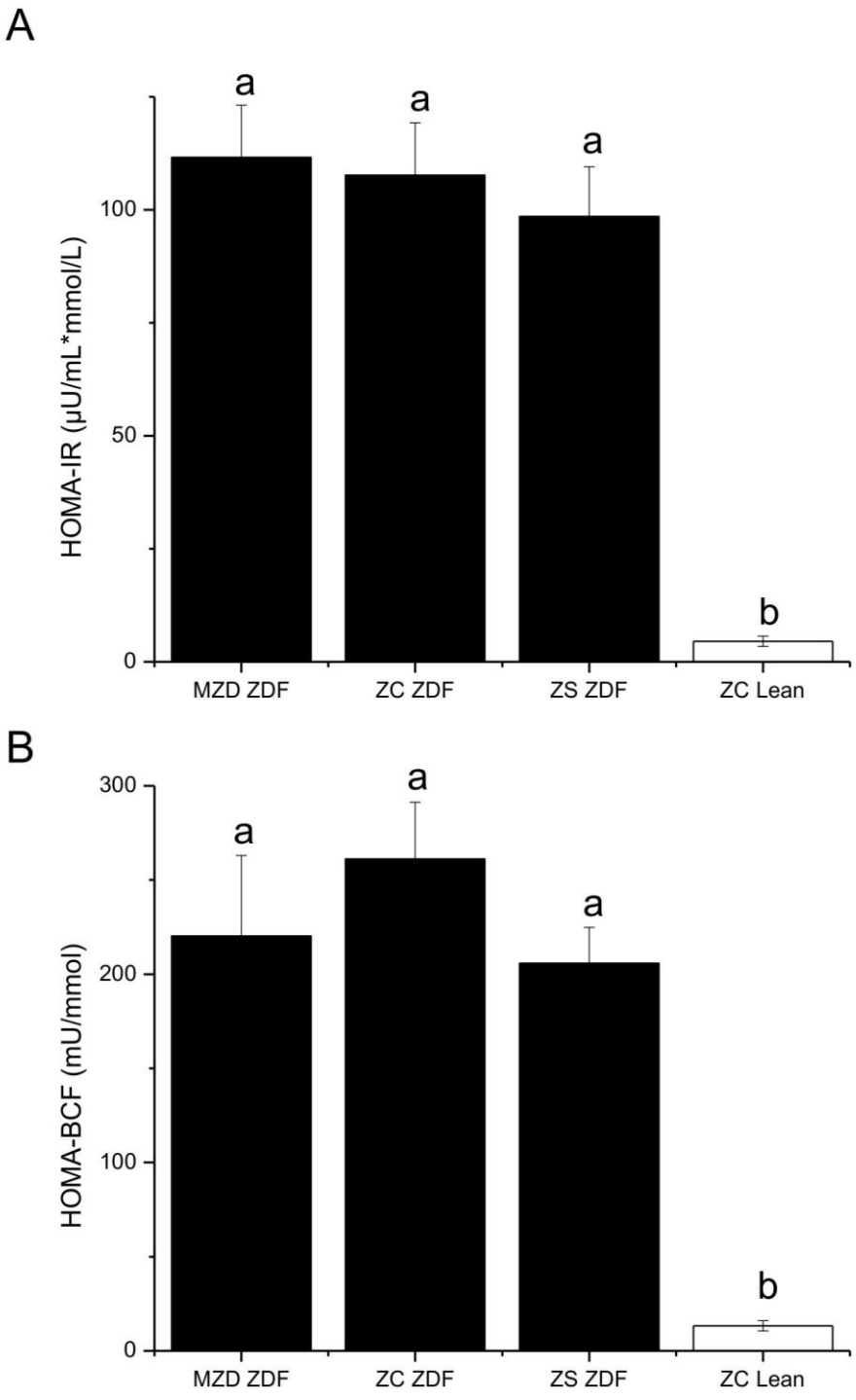


Figure 11. HOMA-IR (A) and HOMA-BCF (B) at termination. Data expressed as means ± SEM, n = 10 except for ZC Lean and ZS where n = 7 and 9, respectively. Different letters indicate significant differences ($P < 0.05$) among means. HOMA-BCF data were log transformed prior to ANOVA.

Pancreatic Function

Pancreas weight, both absolute and adjusted for body weight, was reduced in ZDF rats compared to lean rats (Table 11). ZDF rats had higher HOMA-BCF scores, indicating reduced beta-cell function, at 0 and 4 weeks of the study, compared to lean rats (Figure 12). At 8 weeks, only MZD ZDF rats had an increased HOMA-BCF score compared to lean rats (Figure 12). There was a negative correlation between HOMA-BCF at 8 weeks and pancreas mass ($r = -0.39$, $P < 0.05$) (Figure 13). Representative images of insulin immunostaining for islet cells in pancreas tissue are shown in Figure14. MZD had a greater number of islet cells compared to ZS and lean rats (Table 11, Figure 14). Islet cell area was increased in MZD by 2-fold compared to ZC, 4-fold compared to ZS, and 13-fold compared to lean rats (Figure 14, Figure 15**).** ZC and ZS had a similar number and size of islet cells compared to lean rats (Figure 14).

Table 11. Pancreas Mass and Number of Islet Cells

	ZDF			
	MZD	**ZC**	**ZS**	**ZC Lean**
Pancreas (g)	0.99 ± 0.05[b]	0.90 ± 0.06[b]	1.03 ± 0.05[b]	1.46 ± 0.04[a]
Pancreas (g/100 g BW)[1]	0.234 ± 0.011[b]	0.214 ± 0.016[b]	0.245 ± 0.012[b]	0.457 ± 0.015[a]
Number of Islet Cells (per tissue section)	16.1 ± 2.4[a]	11.4 ± 1.8[ab]	9.7 ± 1.2[b]	7.3 ± 2.1[b]

Values are means ± SEM, n = 10 except for ZC Lean and ZS where n = 7 and 9, respectively. Different superscript letters indicate significant differences ($P < 0.05$) among means. MZD = ZDF fed marginal zinc deficient diet, ZC = ZDF fed zinc control diet, ZS = ZDF fed zinc supplemented diet, ZC Lean = lean ZDF (+/?) fed zinc control diet.

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

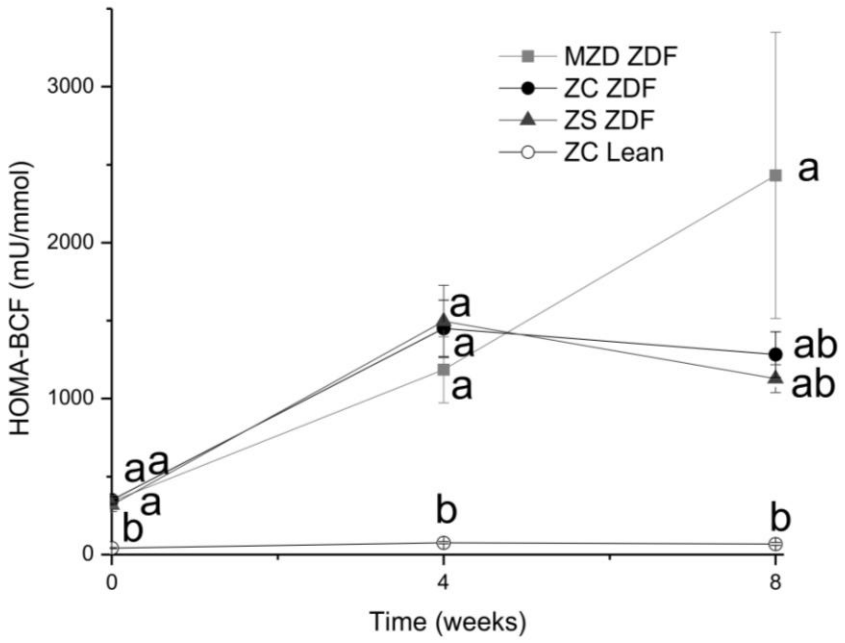


Figure 12. HOMA-BCF at weeks 0, 4, and 8. Data expressed as means ± SEM, n = 10 except for ZC Lean and ZS where n = 7 and 9, respectively. There were significant main effects of time and time × diet interaction. Different letters indicate significant differences ($P < 0.05$) among means at the same time point. MZD = ZDF fed marginal zinc deficient diet, ZC = ZDF fed zinc control diet, ZS = ZDF fed zinc supplemented diet, ZC Lean = lean ZDF (+/?) fed zinc control diet.

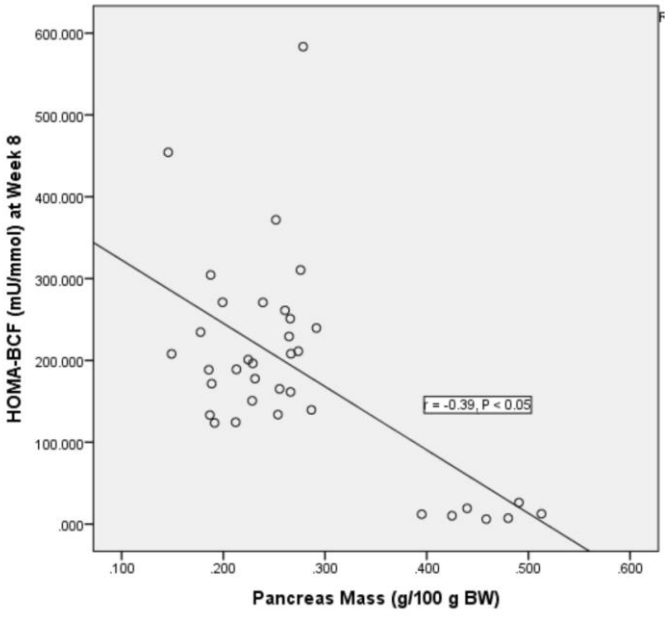


Figure 13. Correlation between HOMA-BCF at week 8 and pancreas mass. n = 36

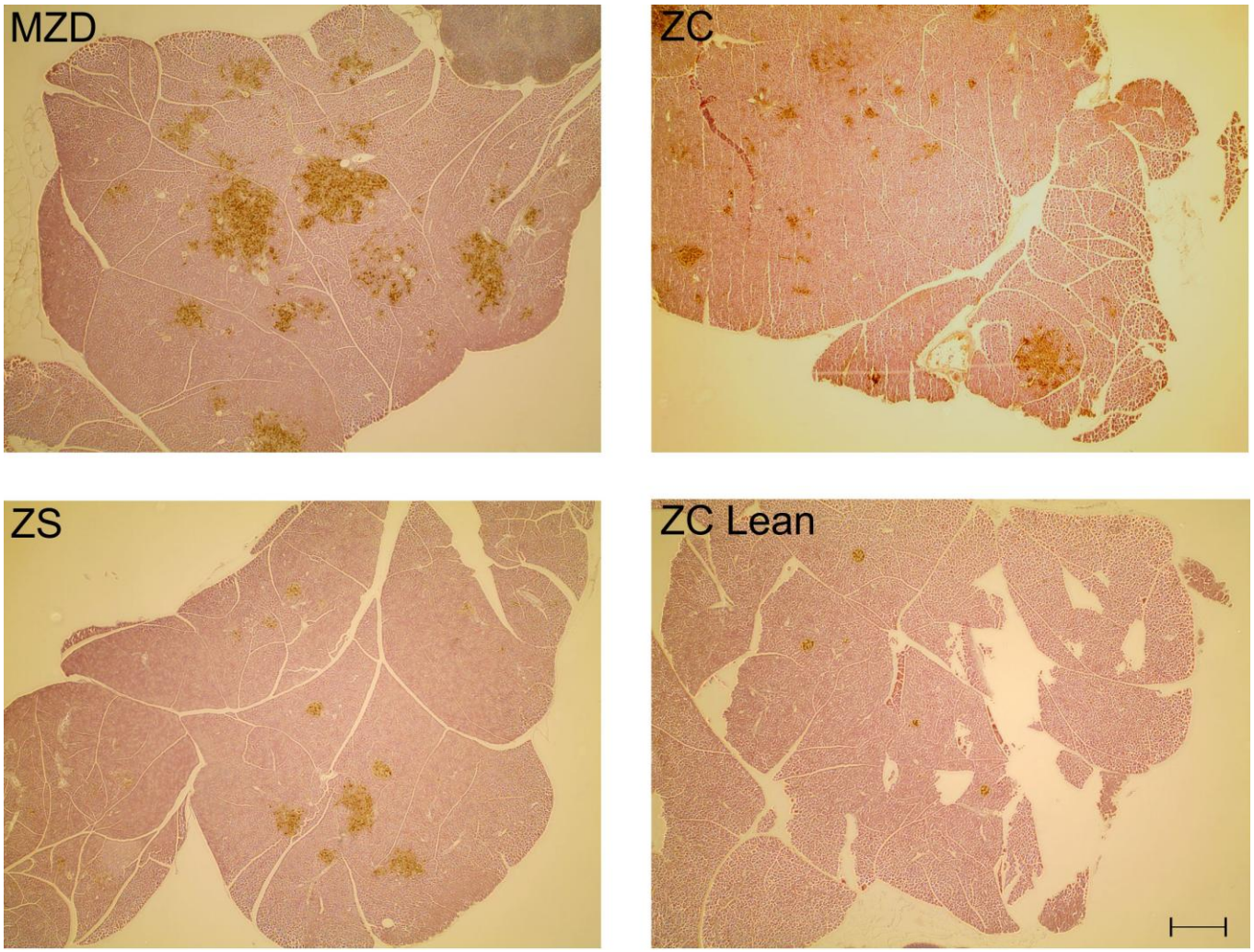


Figure 14. Pancreatic islet insulin immunostaining. Representative images of pancreatic islet cells identified by brown colour from DAB staining. Scale bar equals 0.5 mm.

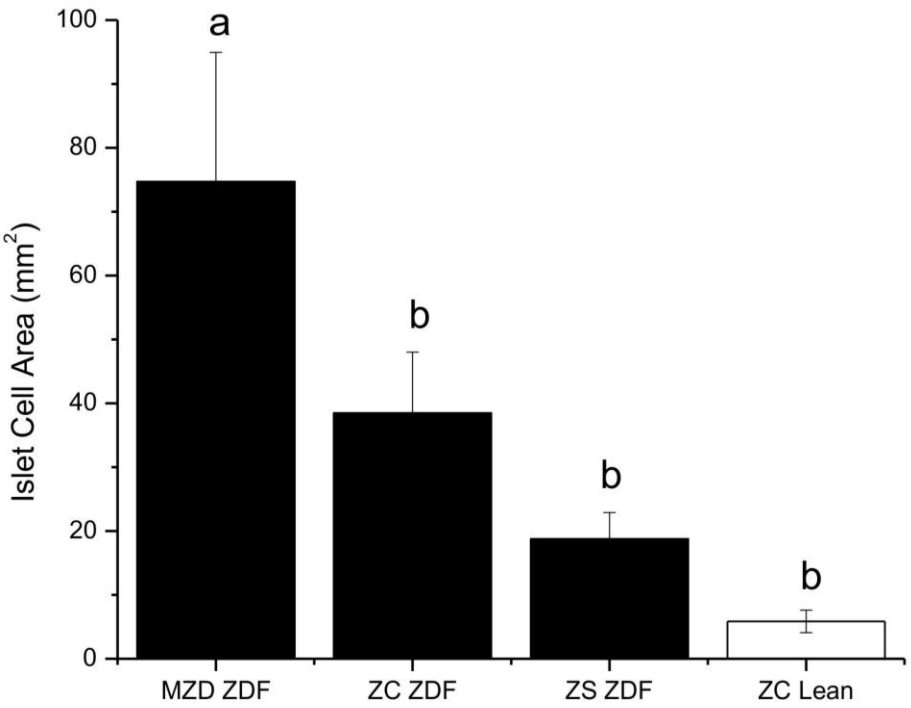


Figure 15. Islet cell area. Data expressed as means ± SEM, n = 10 except for ZC Lean and ZS where n = 7 and 9, respectively. Different letters indicate significant differences ($P < 0.05$) among means. Non-parametric testing was used for analysis.

Liver Function and Circulating Lipids

ZS reduced absolute liver weight compared to ZC in ZDF rats but after adjusting for body weight, liver weight was not different among dietary zinc ZDF groups (Table 12). ZDF rats had higher absolute and adjusted liver weights compared to lean rats (Table 12). ZDF rats had 2.5-fold greater liver total lipid concentrations compared to lean rats (Figure 16).

Fasting serum total cholesterol concentration was elevated in ZDF rats compared to lean rats (Figure 17). Fasting serum LDL-C and free fatty acids was not different between ZDF and lean rats (Figure 17). MZD elevated fasting serum HDL-C compared to all other groups and reduced triglycerides compared to ZC ZDF and ZS ZDF rats (Figure 17).

Table 12. Liver Weight

	ZDF			
	MZD	**ZC**	**ZS**	**ZC Lean**
Liver (g)[1]	20.6 ± 0.7[ab]	22.4 ± 0.9[a]	20.0 ± 0.5[b]	10.6 ± 0.2[c]
Liver (g/100 g BW)[1]	4.88 ± 0.15[a]	5.31 ± 0.24[a]	4.78 ± 0.15[a]	3.32 ± 0.06[b]

Values are means ± SEM, n = 10 except for ZC Lean and ZS where n = 7 and 9, respectively. Different superscript letters indicate significant differences ($P < 0.05$) among means. MZD = ZDF fed marginal zinc deficient diet, ZC = ZDF fed zinc control diet, ZS = ZDF fed zinc supplemented diet, ZC Lean = lean ZDF (+/?) fed zinc control diet. BW = body weight.
[1]Data were log transformed prior to ANOVA.

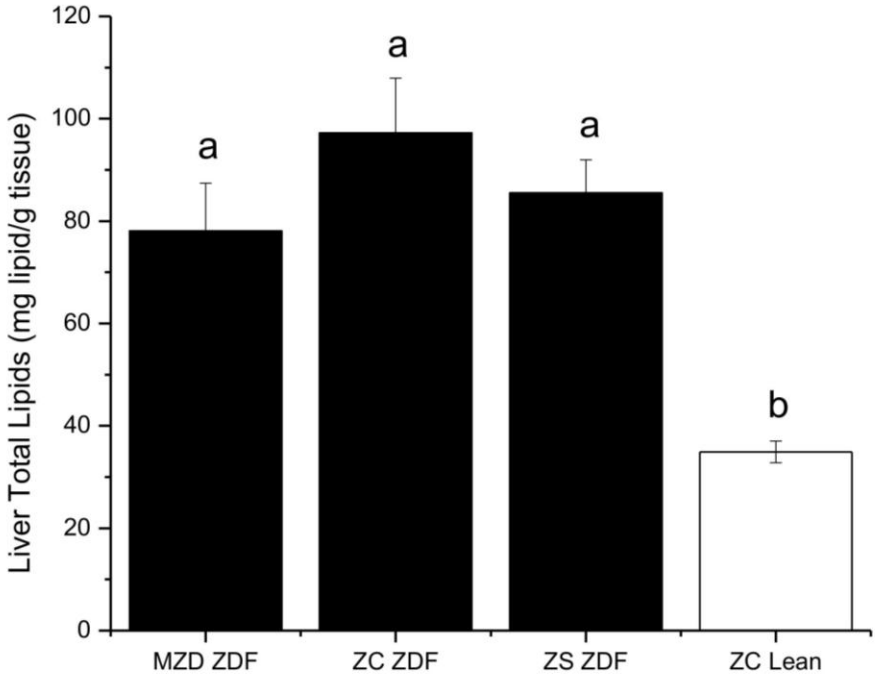


Figure 16. Liver total lipid concentration. Data expressed as means ± SEM, n = 10 except for ZC Lean and ZS where n = 7 and 9, respectively. Different letters indicate significant differences ($P < 0.05$) among means.

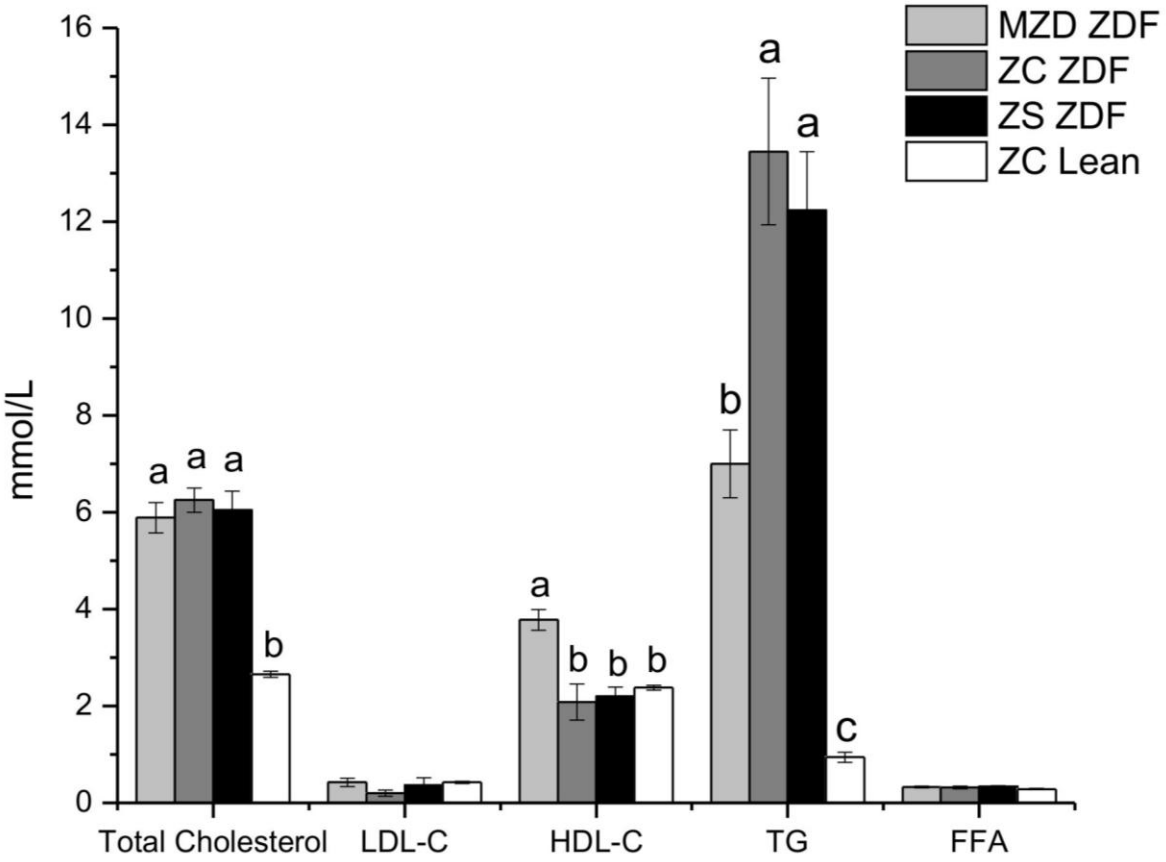


Figure 17. Fasting serum total cholesterol, LDL-C, HDL-C, TG, and FFA. Data expressed as means ± SEM, n = 10 except for ZC Lean and ZS where n = 7 and 9, respectively. Different letters indicate significant differences ($P < 0.05$) among means. Total cholesterol and TG data were log transformed prior to ANOVA. Non-parametric testing was used for HDL-C analysis.

Renal Function

ZDF rats had higher absolute kidney weights compared to lean rats but dietary zinc did not affect kidney weight (left or right kidney) of ZDF rats (Table 13). After adjusting for body weight, kidney weight did not differ between ZDF and lean rats (Table 13). There was no difference in absolute (Table 14) or adjusted for body weight (Figure 18) urine output between ZDF and lean rats throughout the 8 week study. ZDF rats had higher urinary glucose excretion compared to lean rats (Figure 19A). ZS enhanced urinary glucose excretion (adjusted for urine volume) compared to all other groups (Figure 19A). Urinary urea excretion (adjusted for urine volume) was elevated in ZDF rats compared to lean rats (Figure 19B), but creatinine excretion was not different between ZDF and lean rats (Table 15). ZDF rats had 2-fold higher urinary urea to creatinine ratios (adjusted for urine volume) compared to lean rats (Table 15). ZC and ZS had reduced serum creatinine compared to lean rats (Figure 20A). ZS elevated serum urea concentrations by 16% and 23%, compared to MZD and lean rats, respectively (Figure 20B). Reduced serum creatinine and elevated serum urea in the ZS group resulted in a 28% and 55% increase in the urea to creatinine ratio compared to MZD and lean rats, respectively (Figure 20C). Creatinine clearance was not different between ZDF and lean rats (Table 15).

Table 13. Kidney Weights

	ZDF			
	MZD	**ZC**	**ZS**	**ZC Lean**
Left Kidney (g)	1.47 ± 0.04[a]	1.45 ± 0.03[a]	1.47 ± 0.06[a]	1.24 ± 0.03[b]
Left Kidney (g/100 g BW)	0.349 ± 0.001	0.345 ± 0.001	0.352 ± 0.001	0.388 ± 0.001
Right Kidney (g)[1]	1.47 ± 0.04[a]	1.45 ± 0.03[a]	1.47 ± 0.06[a]	1.24 ± 0.03[b]
Right Kidney (g/100 g BW)	0.361 ± 0.001	0.374 ± 0.001	0.382 ± 0.002	0.394 ± 0.001

Values are means ± SEM, n = 10 except for ZC Lean and ZS where n = 7 and 9, respectively. Different superscript letters indicate significant differences ($P < 0.05$) among means. MZD = ZDF fed marginal zinc deficient diet, ZC = ZDF fed zinc control diet, ZS = ZDF fed zinc supplemented diet, ZC Lean = lean ZDF (+/?) fed zinc control diet. BW = body weight.

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

Table 14. Absolute Urine Output at Weeks 0, 4, and 8

Urine Output (mL/5 hrs)	ZDF			ZC Lean
	MZD	**ZC**	**ZS**	
Week 0	3.20 ± 0.24	3.06 ± 0.27	3.43 ± 0.42	1.67 ± 0.28
Week 4	2.23 ± 0.33	4.02 ± 0.95	2.73 ± 0.41	2.76 ± 0.41
Week 8	3.23 ± 0.49	3.96 ± 0.50	3.51 ± 0.52	3.67 ± 0.56

Values are means ± SEM, n = 10 except for ZC Lean and ZS where n = 7 and 9, respectively. Absence of letters indicates no significant differences (P > 0.05) among means. MZD = ZDF fed marginal zinc deficient diet, ZC = ZDF fed zinc control diet, ZS = ZDF fed zinc supplemented diet, ZC Lean = lean ZDF (+/?) fed zinc control diet.

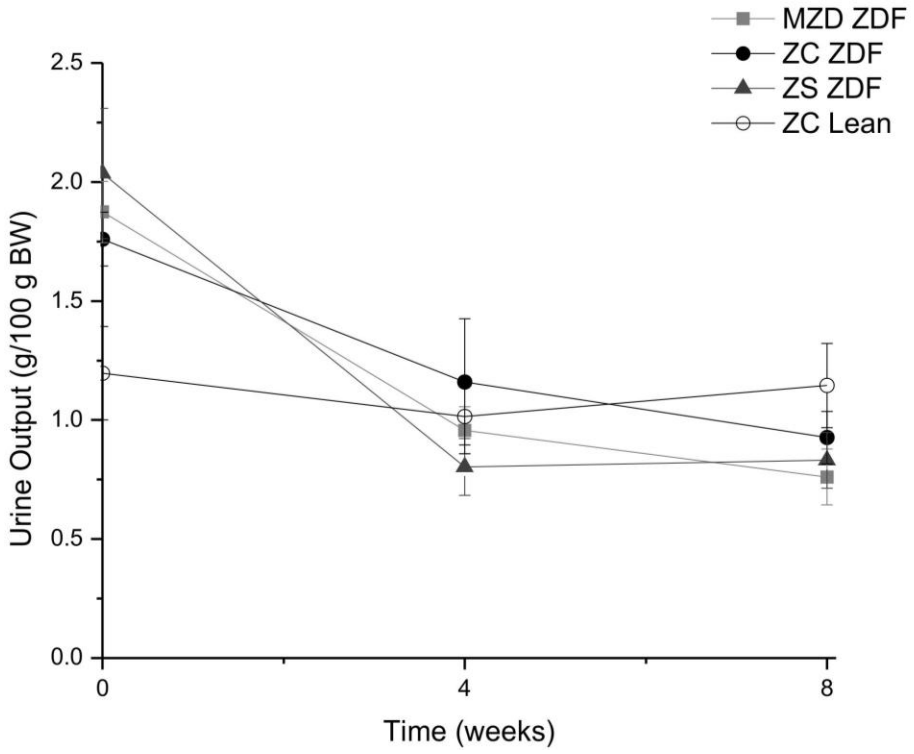


Figure 18. Urine output at weeks 0, 4, and 8. Data expressed as means ± SEM, n = 10 except for ZC Lean and ZS where n = 7 and 9, respectively. Absence of letters indicates no significant differences (P > 0.05) among means at the same time point. There were no significant main effects of time and time × diet interaction. BW = body weight.

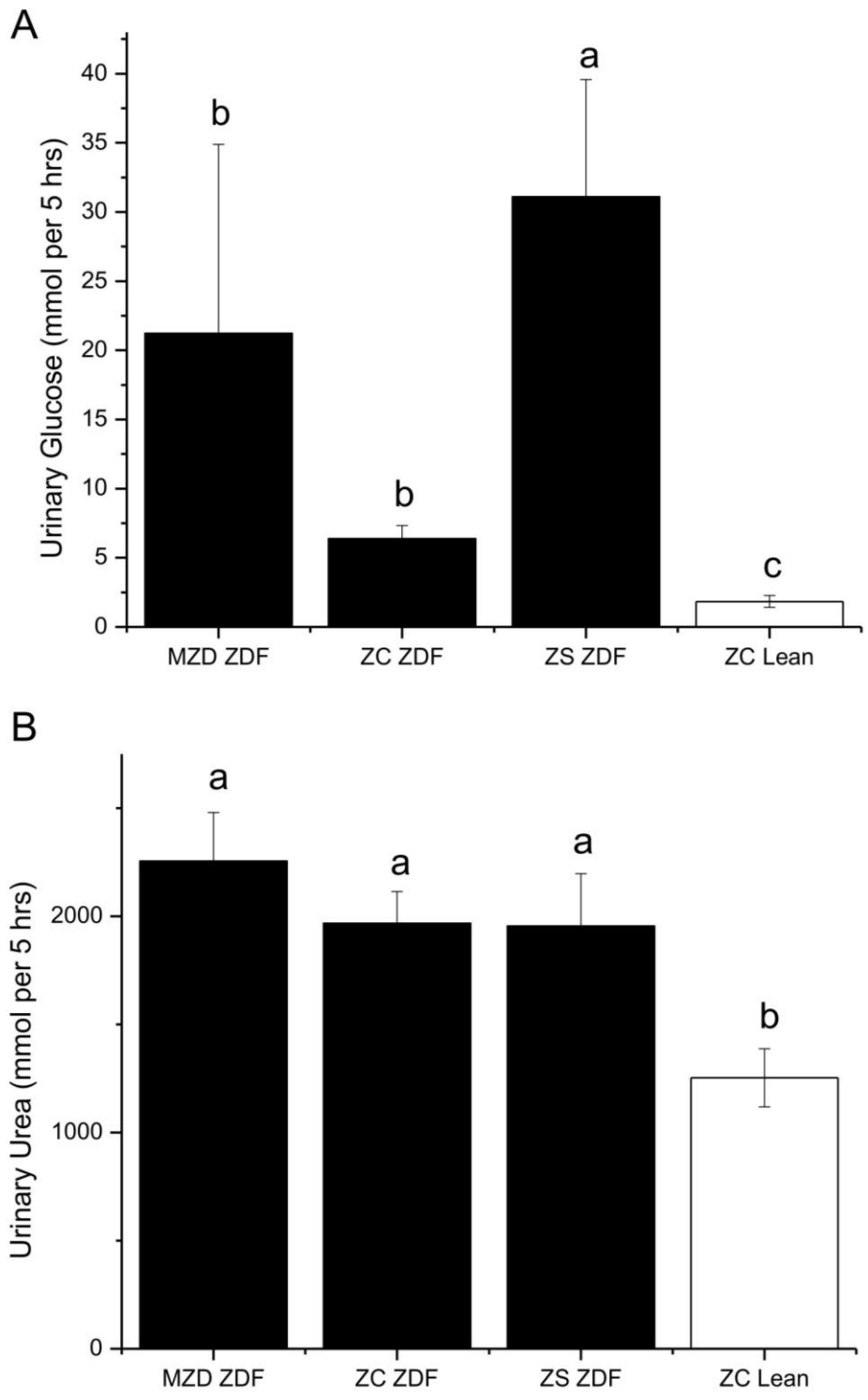


Figure 19. Urinary glucose (A) and urea (B) concentrations. Data expressed as means ± SEM, n = 8 except for ZC Lean and ZC where n = 7 and 9, respectively. Different letters indicate significant differences ($P < 0.05$) among means. Data were log transformed prior to ANOVA.

Table 15. Urinary Metabolite Excretion

	ZDF			
	MZD	**ZC**	**ZS**	**ZC Lean**
Urine				
Creatinine (mmol per 5 hr)	12.1 ± 0.95	10.8 ± 0.79	10.0 ± 1.15	12.9 ± 1.05
Urea:Creatinine Ratio (mmol per 5 hrs:mmol per 5 hrs)	185 ± 6[a]	184 ± 10[a]	196 ± 14[a]	96 ± 4[b]
Creatinine Clearance (mL/min)	1.39 ± 0.14	1.33 ± 0.13	1.27 ± 0.14	1.32 ± 0.12

Values are means ± SEM, n = 9 for MZD and ZS, n = 10 for ZC, and n = 7 for ZC Lean. Different superscript letters indicate significant differences ($P < 0.05$) among means. MZD = ZDF fed marginal zinc deficient diet, ZC = ZDF fed zinc control diet, ZS = ZDF fed zinc supplemented diet, ZC Lean = lean ZDF (+/?) fed zinc control diet.

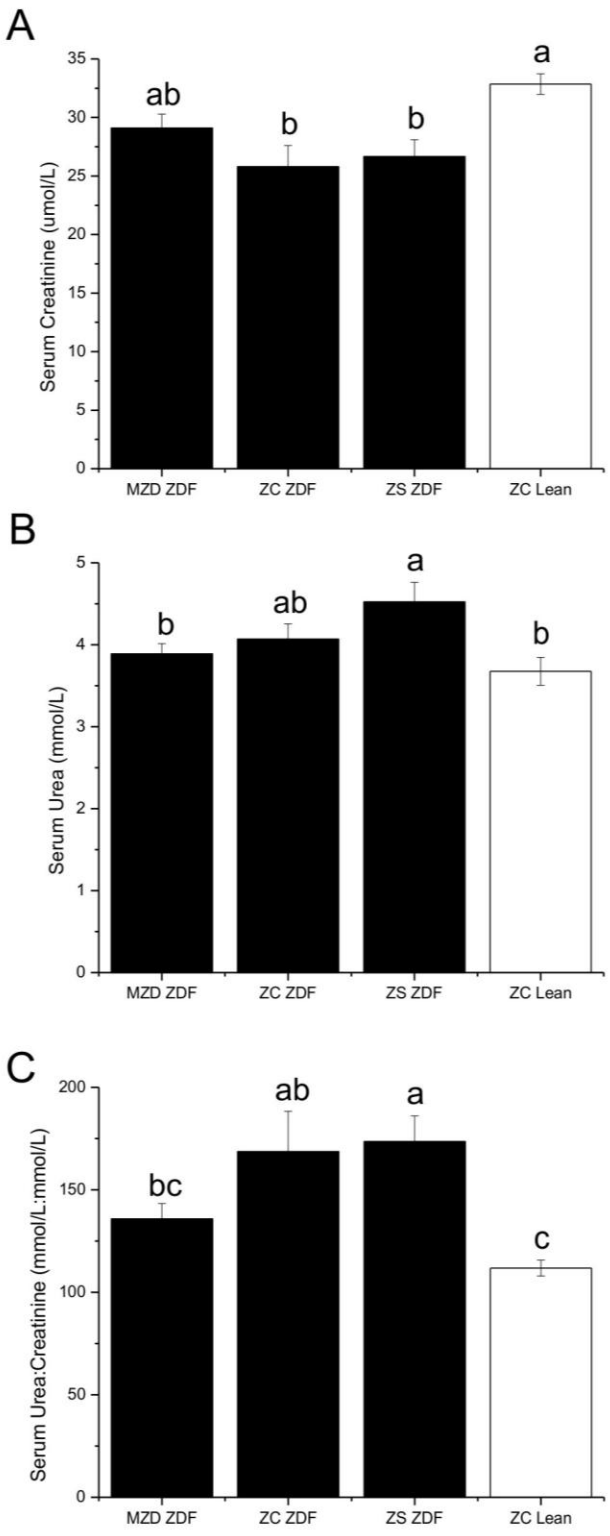


Figure 20. Serum creatinine (A), urea (B), and urea to creatinine ratio (C). Data expressed as means ± SEM, n = 10 except for ZC Lean and ZS where n = 7 and 9, respectively. Different letters indicate significant differences ($P < 0.05$) among means. Serum urea to creatinine ratio was log transformed prior to ANOVA.

Zinc Status

Serum Zinc Concentrations

ZS elevated serum zinc concentrations and total zinc in the circulation compared to all other ZDF groups and lean rats (Table 16). ZDF rats had higher total serum zinc content than lean rats (Table 16).

Femur Zinc Concentrations

Femur zinc concentrations were enhanced by 41% and 59% in ZS ZDF and reduced by 56% and 51% in MZD ZDF compared to ZC and ZC lean, respectively (Figure 21).

Urinary Zinc Excretion

At baseline (week 0), ZDF rats were excreting an average of 19.9 ± 5.3 µmol of zinc per 5 hours or 2.85 ± 0.60 µmol/100 g body weight, while at week 8, ZDF rats were excreting an average of 44.9 ± 8.0 µmol of zinc per 5 hours or 2.80 ± 0.44 µmol/100 g body weight. At week 8, MZD ZDF and lean rats were excreting less zinc in the urine than ZC ZDF and ZS ZDF rats (Table 16). ZDF rats fed ZC had elevated zinc excretion at week 8 compared to week 0 (45.4 ± 7.0 vs. 5.27 ± 1.21 µmol per 5 hours); ZS elevated (although it was not statistically significant) while MZD reduced urinary zinc excretion at week 8 compared to ZC. MZD ZDF had lower urinary zinc excretion (µg/mg creatinine and µmol/100 g body weight), than ZC ZDF and ZS ZDF rats. Meanwhile, lean rats had the lowest urinary zinc excretion (µg/mg creatinine) (Table 16).

Table 16. Serum and Urinary Zinc Concentrations at Week 8

	ZDF			
	MZD	**ZC**	**ZS**	**ZC Lean**
Serum Zinc (µmol/L)[1]	40.5 ± 1.3[b]	44.0 ± 1.1[b]	53.9 ± 2.3[a]	41.8 ± 1.32[b]
Total Serum Zinc (µmol/rat)[2]	997 ± 41[b]	1080 ± 32[b]	1310 ± 61[a]	774 ± 26[c]
Urine Zinc at Week 8 (µmol per 5 hrs)[1, 3]	31.3 ± 19.4[b]	45.4 ± 7.0[a]	59.5 ± 15.9[a]	7.5 ± 3.9[b]
Urine Zinc at Week 8 (µg/mg creatinine)[1, 4]	0.37 ± 0.11[b]	2.40 ± 0.41[a]	4.71 ± 1.13[a]	0.17 ± 0.08[c]
Urine Zinc at Week 8 (µmol/100 g BW)[1, 3]	1.76 ± 0.99[b]	3.30 ± 0.69[a]	3.35 ± 0.56[a]	0.65 ± 0.89[b]

Values are means ± SEM, n = 9 for MZD, n = 10 for ZC, n = 7 for ZC Lean, and n = 8 for ZS. Different superscript letters indicate significant differences ($P < 0.05$) among means. MZD = ZDF fed marginal zinc deficient diet, ZC = ZDF fed zinc control diet, ZS = ZDF fed zinc supplemented diet, ZC Lean = lean ZDF (+/?) fed zinc control diet. BW = body weight.

[1]Data were log transformed prior to analysis.

[2]Total serum zinc content was calculated by adjusting for total blood volume [58 mL blood volume/kg body weight (Olfert, Cross, & McWillam, 1993)].

[3]n = 9 for MZD, n = 10 for ZC, n = 8 for ZS, and n = 6 for ZC lean.

[4]n = 9 for MZD, n = 10 for ZC, n = 9 for ZS, and n = 6 for ZC lean.

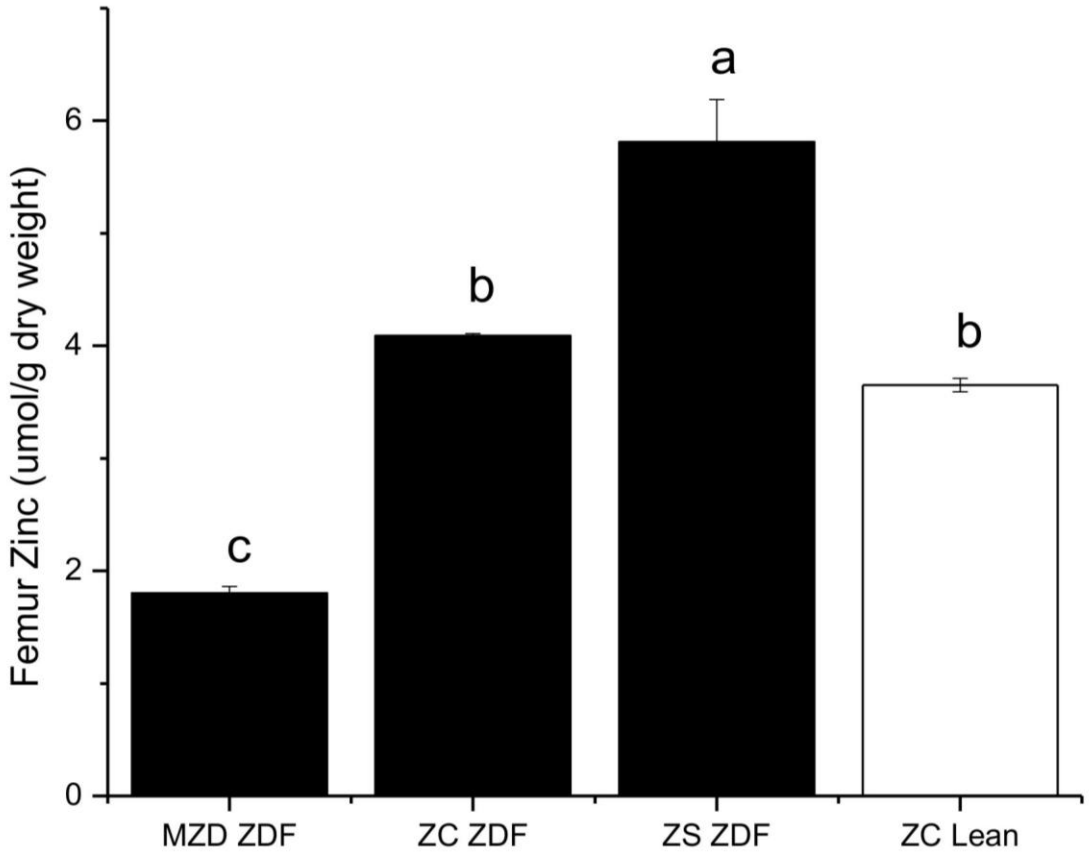


Figure 21. Femur zinc concentrations. Data expressed as means ± SEM, n = 10 except for ZC Lean and ZS where n = 7 and 9, respectively. Different letters indicate significant differences ($P < 0.05$) among means. Non-parametric testing was used for statistical analysis.

Pancreatic Zinc Concentrations

MZD reduced pancreatic zinc concentrations compared to all other dietary ZDF groups and lean rats (Figure 22). ZC ZDF rats had 25% lower pancreas zinc concentrations than ZC lean rats (Figure 22). ZS elevated pancreatic zinc concentrations of ZDF to the level of lean rats (Figure 22).

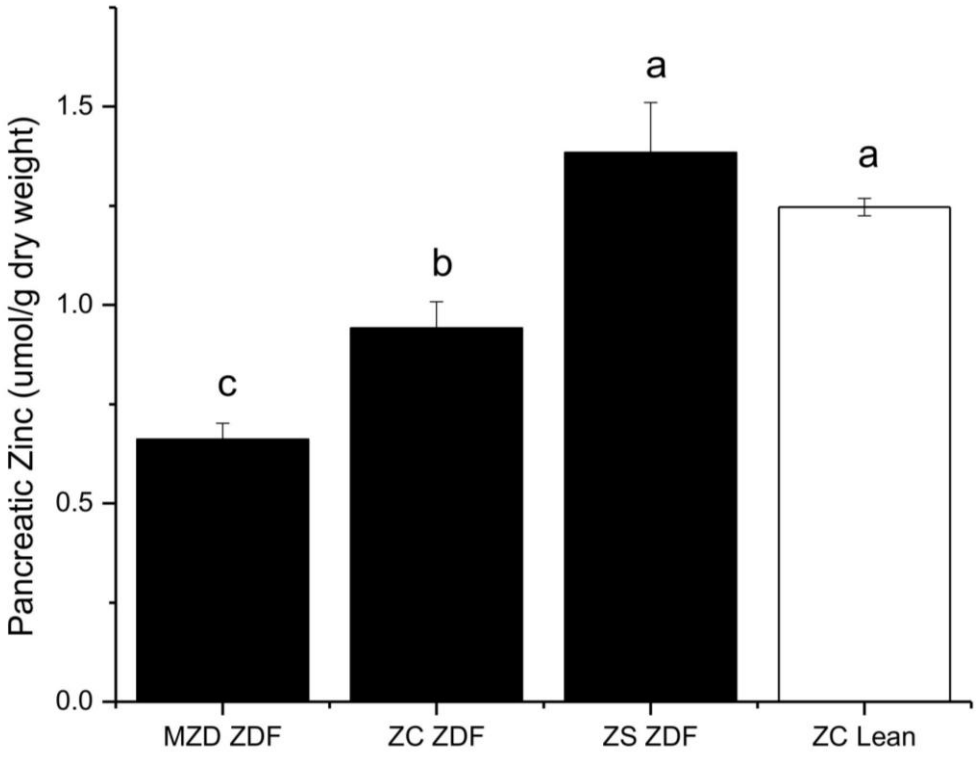


Figure 22. Pancreatic Zinc Concentrations. Data expressed as means ± SEM, n = 10 except for ZC Lean and ZS where n = 7 and 9, respectively. Different letters indicate significant differences ($P < 0.05$) among means.

Epididymal Fat Zinc Concentrations

There was a trend for MZD ZDF to have higher epididymal fat zinc concentrations than lean rats (Table 17).

Table 17. Epididymal Fat Zinc Concentrations

	ZDF			
	MZD	**ZC**	**ZS**	**ZC Lean**
Zinc (µmol/g dry weight)[1]	0.190 ± 0.046^{a}	0.127 ± 0.020^{ab}	0.148 ± 0.035^{ab}	0.086 ± 0.009^{b}

Values are means ± SEM, n = 9 for MZD, n = 10 for ZC, n = 8 for ZS, and n = 7 for ZC Lean. Different superscript letters indicate a trend for significant differences (P = 0.11) among means. MZD = ZDF fed marginal zinc deficient diet, ZC = ZDF fed zinc control diet, ZS = ZDF fed zinc supplemented diet, ZC Lean = lean ZDF (+/?) fed zinc control diet.

[1]Data was log transformed prior to ANOVA.

Epididymal Fat Zinc Transporters

Epididymal fat protein levels of ZnT3 were not different between dietary zinc groups or between ZDF and lean rats (Table 18, Figure 23).

Table 18. Epididymal Fat Protein Levels of ZnT3

	ZDF			
	MZD	**ZC**	**ZS**	**ZC Lean**
ZnT3	1.32 ± 0.19	1.25 ± 0.15	1.35 ± 0.26	1.43 ± 0.32

Values are means ± SEM, n = 10 except for ZC Lean and ZS where n = 7 and 9, respectively. ZnT3 was analyzed by Western blotting. Relative intensities of the bands were quantified by a densitometer. All data were adjusted by the loading control (β-actin) to control for differences in protein loading. Data expressed in arbitrary units. Absence of superscript letters indicates no significant differences (P > 0.05) among means. MZD = ZDF fed marginal zinc deficient diet, ZC = ZDF fed zinc control diet, ZS = ZDF fed zinc supplemented diet, ZC Lean = lean ZDF (+/?) fed zinc control diet.

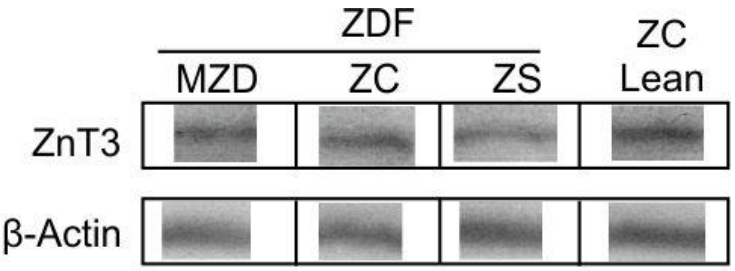


Figure 23. Representative blots of ZnT3. Figure was obtained from Western Blot analyses.

Liver Zinc Concentrations

Liver zinc concentrations (µmol/g dry weight) were lower but total zinc content (µg/wet liver) was 1.7-fold higher in ZDF compared to lean rats (Table 19). Relative liver

zinc content (μg/g wet liver) was lower in MZD ZDF and ZC ZDF than lean rats (Table 19).

Liver Mineral Concentrations

Liver Copper Concentrations

Liver copper concentrations were greatly reduced (by 40%) in ZS ZDF, meanwhile, MZD ZDF and ZC ZDF had only slightly lower copper concentrations than lean rats (Table 19). ZS reduced relative liver copper content compared to all other groups (Table 19). Total copper content was reduced in ZS ZDF compared to MZD ZDF and ZC ZDF, although ZDF rats had higher total copper content than lean rats (Table 19).

Liver Iron Concentrations

Iron concentrations were reduced in ZC ZDF and ZS ZDF compared to lean rats (Table 19). Relative liver iron content was not different between ZDF and lean rats, while total copper content was elevated by 1.7-fold in ZDF compared to lean rats (Table 19).

Table 19. Liver Mineral Concentrations and Content

	ZDF			
	MZD	**ZC**	**ZS**	**ZC Lean**
Zinc (μmol/g dry weight)[1]	1.02 ± 0.03[b]	1.01 ± 0.05[b]	1.06 ± 0.05[b]	1.45 ± 0.04[a]
Relative Zinc Content (μg/g wet liver)	21.2 ± 0.9[b]	21.8 ± 1.1[b]	22.5 ± 1.2[ab]	25.5 ± 0.9[a]
Total Zinc Content (μg/wet liver)[1]	434 ± 16[a]	480 ± 14[a]	445 ± 20[a]	269 ± 8[b]
Copper (μmol/g dry weight)	0.215 ± 0.011[b]	0.203 ± 0.010[b]	0.148 ± 0.010[c]	0.259 ± 0.018[a]
Relative Copper Content (μg/g wet liver)	4.37 ± 0.25[a]	4.31 ± 0.32[a]	3.04 ± 0.20[b]	4.39 ± 0.33[a]
Total Copper Content (μg/wet liver)	88.8 ± 2.9[a]	94.3 ± 4.2[a]	60.2 ± 3.5[b]	46.2 ± 2.9[c]
Iron (μmol/g dry weight)	9.66 ± 0.85[ab]	7.61 ± 0.69[b]	8.25 ± 0.91[b]	11.00 ± 0.60[a]
Relative Iron Content (μg/g wet liver)	180 ± 27	139 ± 12	154 ± 23	164 ± 11
Total Iron Content (μg/wet liver)[1]	3593 ± 416[a]	3032 ± 167[a]	2999 ± 376[a]	1726 ± 98[b]

Values are means ± SEM, n = 10 except for ZC Lean and ZS where n = 7 and 9, respectively. Zinc, copper and iron were measured using ICP-OES. Different superscript letters indicate significant differences ($P < 0.05$) among means. MZD = ZDF fed marginal zinc deficient diet, ZC = ZDF fed zinc control diet, ZS = ZDF fed zinc supplemented diet, ZC Lean = lean ZDF (+/?) fed zinc control diet.
[1]Data were log transformed prior to ANOVA.

Femur Mineral Concentrations

Total femur area and bone mineral content, as measured by DEXA scanning, were lower in ZDF compared to lean rats (Table 20). Bone mineral density was not different between ZDF and lean rats (Table 20). Calcium and phosphorus concentrations were not altered by dietary zinc intake and were not different between ZDF and lean rats (Table 20).

Table 20. Femur Mineral Concentrations, Content and Density

	ZDF			
	MZD	**ZC**	**ZS**	**ZC Lean**
Total Area (cm^2)	1.67 ± 0.03^b	1.64 ± 0.03^b	1.65 ± 0.02^b	1.82 ± 0.01^a
Bone Mineral Content (g)	0.336 ± 0.008^b	0.324 ± 0.008^b	0.326 ± 0.006^b	0.363 ± 0.004^a
Bone Mineral Density (g/cm^2)	0.202 ± 0.002	0.200 ± 0.002	0.198 ± 0.002	0.200 ± 0.002
Calcium (µmol/g dry weight)	6377 ± 377	6350 ± 342	5997 ± 40	5963 ± 47
Phosphorus (µmol/g dry weight)	3721 ± 39	3733 ± 10	3706 ± 24	3686 ± 33

Values are means ± SEM, n = 9 for MZD, n = 10 for ZC, n = 7 for ZC Lean, and n = 8 for ZS, except n= 8 for MZD and n = 9 for ZC, for femur phosphorus concentrations. Different superscript letters indicate significant differences ($P < 0.05$) among means. MZD = ZDF fed marginal zinc deficient diet, ZC = ZDF fed zinc control diet, ZS = ZDF fed zinc supplemented diet, ZC Lean = lean ZDF (+/?) fed zinc control diet. Total area, bone mineral content, and bone mineral density were analyzed by DEXA. Femur calcium and phosphorus were measured by ICP-OES.

Femur Dimensions

Total femur length was reduced by ZS compared to MZD (Table 21). ZDF rats had shorter femur total length than lean rats (Table 21). Femur width at the hip joint was reduced in ZC and ZS ZDF rats compared to lean rats (Table 21). The width of the femur at the knee joint, diaphysis (narrowest place), and neck, were not different between ZDF and lean rats (Table 21). ZDF rats had narrower femur heads compared to lean rats (Table 21).

Table 21. Femur Dimensions

	ZDF			
	MZD	**ZC**	**ZS**	**ZC Lean**
Total Length (cm)[1]	3.44 ± 0.03[b]	3.40 ± 0.03[bc]	3.26 ± 0.12[c]	3.66 ± 0.02[a]
Hip Joint (cm)[2]	0.788 ± 0.010[ab]	0.775± 0.005[b]	0.777 ± 0.007[b]	0.813 ± 0.012[a]
Knee Joint (cm)	0.711 ± 0.012	0.700 ± 0.010	0.697 ± 0.010	0.730 ± 0.013
Diaphysis (cm)	0.427 ± 0.034	0.396 ± 0.007	0.405 ± 0.004	0.414 ± 0.007
Neck (cm)	0.216 ± 0.002	0.214 ± 0.002	0.215 ± 0.002	0.222 ± 0.002
Head (cm)	0.369 ± 0.002[b]	0.369 ± 0.002[b]	0.370 ± 0.003[b]	0.385 ± 0.003[a]

Values are means ± SEM, n = 9 for MZD, n = 10 for ZC, n = 7 for ZC Lean, and n = 8 for ZS. Different superscript letters indicate significant differences ($P < 0.05$) among means. MZD = ZDF fed marginal zinc deficient diet, ZC = ZDF fed zinc control diet, ZS = ZDF fed zinc supplemented diet, ZC Lean = lean ZDF (+/?) fed zinc control diet. Femur dimensions were measured using callipers and all measurements were done in triplicate.

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

[2]Data was log transformed prior to ANOVA.

Inflammation

Local Inflammation

Epididymal fat protein levels of TNF-α (precursor and mature forms) and SOCS-3 were elevated in lean rats compared to ZDF rats (Table 22, Figure 24). Epididymal fat protein levels of MCP-1 were not different between ZDF rats and lean rats (Table 22, Figure 24).

Systemic Inflammation

Circulating concentrations and total serum content of haptoglobin (Figure 25A, B) and MCP-1 (Figure 25C, D) were elevated in ZDF rats compared to lean rats, but there was no effect of zinc on these markers of systemic inflammation.

Table 22. Epididymal Fat Protein Levels of TNF-α, SOCS-3 and MCP-1

	ZDF			
	MZD	**ZC**	**ZS**	**ZC Lean**
TNF-α (precursor)[1]	1.91 ± 0.27[b]	3.37 ± 1.63[b]	1.98 ± 0.30[b]	9.02 ± 2.89[a]
TNF-α (mature)[2]	0.23 ± 0.04[b]	0.48 ± 0.17[b]	0.90 ± 0.43[b]	1.64 ± 0.13[a]
SOCS-3[2]	1.9 ± 0.3[b]	4.2 ± 1.7[b]	1.8 ± 0.2[b]	14.7 ± 2.3[a]
MCP-1	1.01 ± 0.17	2.05 ± 0.75	1.15 ± 0.20	2.70 ± 1.17

Values are means ± SEM. For SOCS-3, n = 10 except for ZC Lean and ZS where n = 6 and 8, respectively. For TNF-α (precursor) and MCP-1, n = 10 except for ZC Lean and ZS where n = 5 and 8, respectively. For TNF-α (mature), n = 8 except for ZC Lean and ZC where n = 7 and 10, respectively. TNF-α, SOCS-3, and MCP-1 were analyzed by Western blotting. Relative intensities of the bands were quantified by a densitometer. All data were adjusted by the loading control (β-actin) to control for differences in protein loading. Data expressed in arbitrary units. Different superscript letters indicate significant differences ($P < 0.05$) among means. MZD = ZDF fed marginal zinc deficient diet, ZC = ZDF fed zinc control diet, ZS = ZDF fed zinc supplemented diet, ZC Lean = lean ZDF (+/?) fed zinc control diet.

[1]Data was log transformed prior to ANOVA.

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

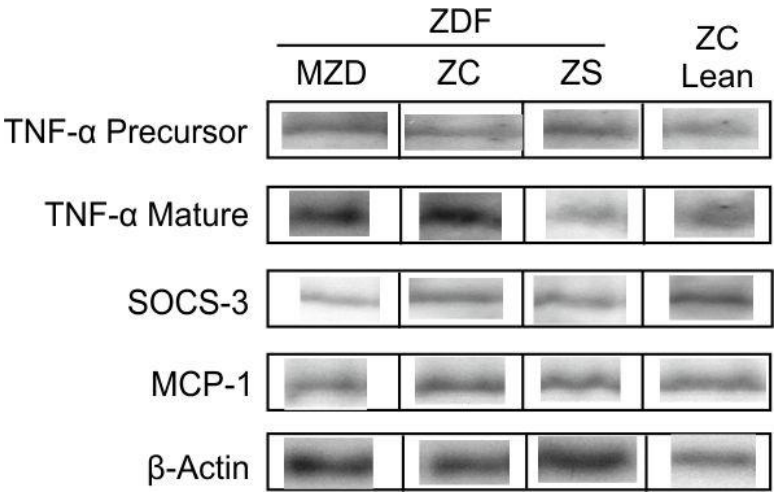


Figure 24. Representative blots of inflammatory proteins. Figures were obtained from Western Blot analyses.

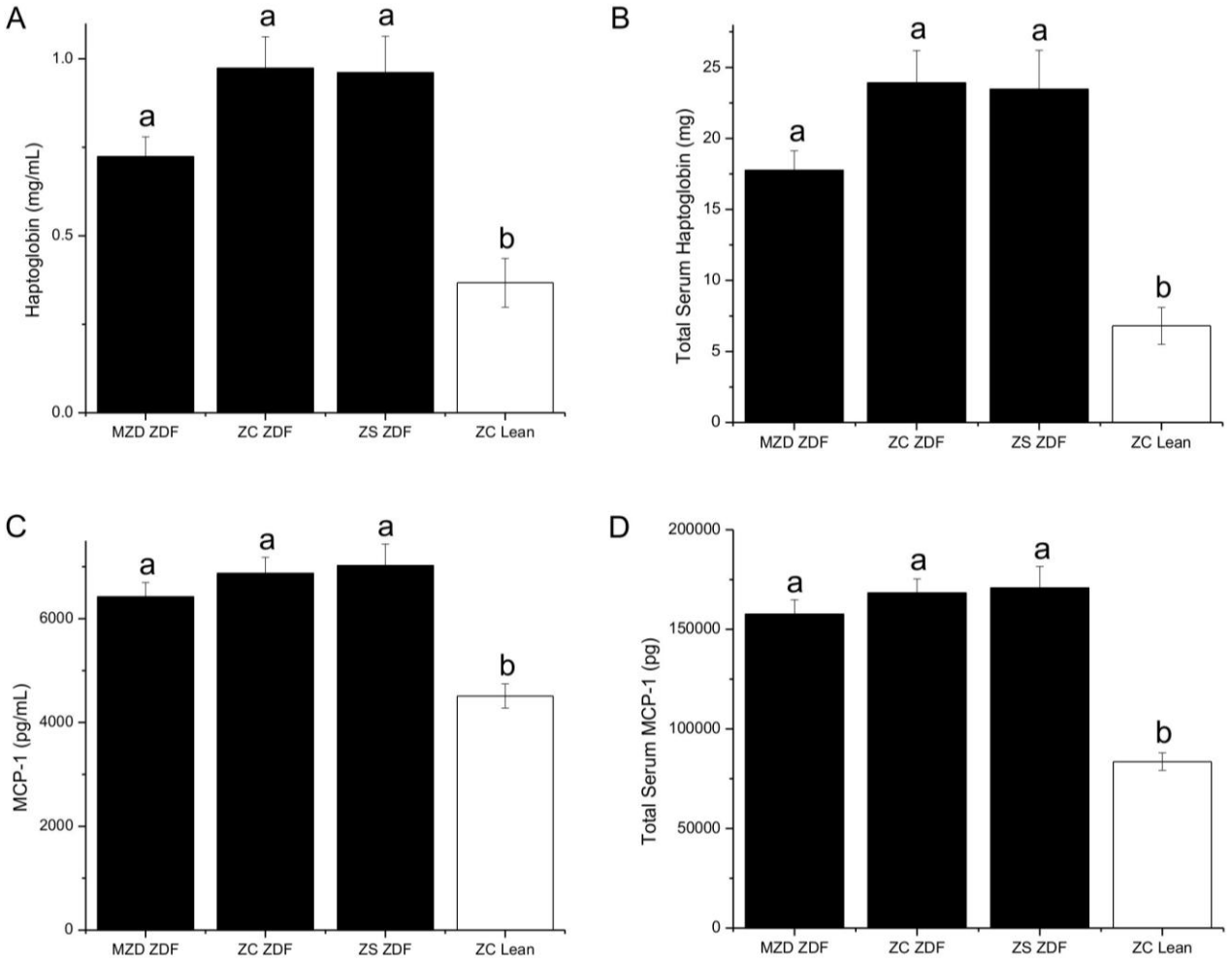


Figure 25. Serum haptoglobin (A, B) and MCP-1 (C, D) concentrations. Data expressed as means ± SEM, n = 10 except for ZC Lean and ZS where n = 7 and 9, respectively. Different letters indicate significant differences ($P < 0.05$) among means.

Circulating Cytokines

At euthanization, there was a trend (P = 0.08) for ZDF rats to have reduced circulating TNF-α concentrations compared to lean rats (Figure 26A).There was a trend for ZS to reduce serum concentrations of IFN-γ (P = 0.06) (Figure 26B) and IL-6 (P = 0.09) (Figure 26C) compared to lean rats (81.3 ± 3.6 pg/mL vs. 103 ± 5 pg/mL, and 54.1 ± 5.5 pg/mL vs. 77.3 ± 6.5 pg/mL, respectively), but circulating concentrations of IL-1β were not different between ZDF and lean rats (Figure 26D). Although, total serum concentrations [calculated by adjusting for blood volume (Olfert, Cross, & McWillam, 1993)] of TNF-α, IFN-γ, and IL-6 were not different between ZDF and lean rats, however IL-1β was higher in ZDF compared to lean rats (Table 23).

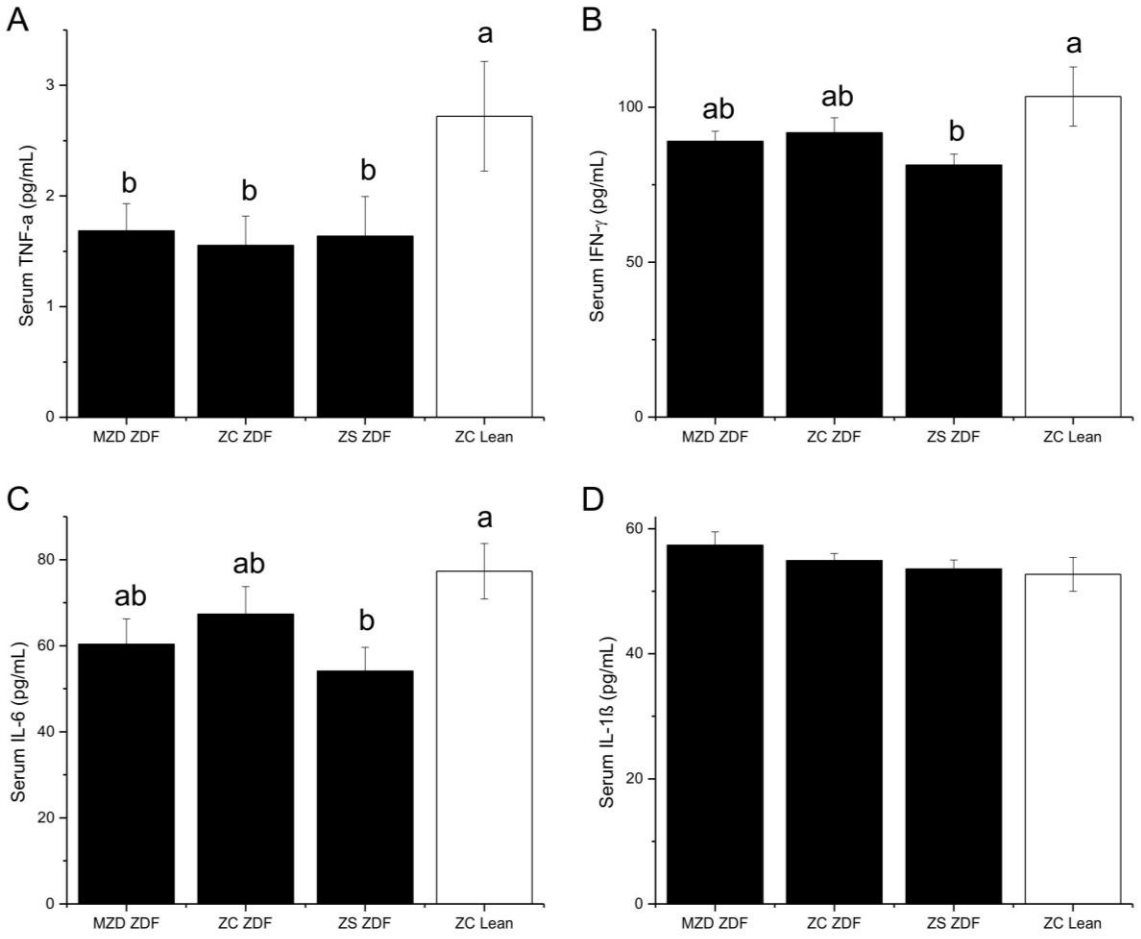


Figure 26. Serum TNF-α (A), IFN-γ (B), IL-6 (C), and IL-1β (D) concentrations. Data expressed as means ± SEM, n = 10 except for ZC Lean and ZS where n = 7 and 6, respectively. Different letters indicate significant differences ($P < 0.05$) among means, except for TNF-α where $P = 0.08$, IFN-γ where $P = 0.06$, and IL-6 where $P = 0.09$. IL-1β data was analyzed using non-parametric statistics.

Table 23. Serum Cytokine Content

	ZDF			
	MZD	**ZC**	**ZS**	**ZC Lean**
TNF-α (pg)	41.1 ± 6.3	38.0 ± 6.4	40.3 ± 8.8	50.2 ± 9.0
IFN-γ (pg)	2188 ± 97	2246 ± 113	1974 ± 93	1916 ± 176
IL-6 (pg)	1481 ± 147	1642 ± 142	1313 ± 134	1431 ± 117
IL-1β (pg)[1]	1405 ± 52[a]	1346 ± 33[a]	1298 ± 32[a]	957 ± 54[b]

Values are means ± SEM, n = 10 except for ZC Lean and ZS where n = 7 and 9, respectively. Total serum content was calculated by adjusting for total blood volume [58 mL blood volume/kg body weight (Olfert, Cross, & McWillam, 1993)]. Superscript letters indicate significant differences ($P < 0.05$) between means. MZD = ZDF marginal zinc deficiency diet, ZC = ZDF zinc control diet, ZS = ZDF zinc supplemented diet, ZC Lean = lean ZDF (+/?) zinc control diet.

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

Immune Function

Lymphoid Organ Weight

Absolute spleen weight was greater in MZD and ZC ZDF rats compared to lean rats (Table 24). Adjusted spleen weight was not different among dietary zinc ZDF rats but was lower in ZDF rats compared to lean rats (Table 24). ZS reduced absolute and adjusted thymus weight compared to MZD and ZC, although ZDF rats had higher thymus weights than lean rats (Table 24).

Immune Cell Proportions

After isolating immune cells from the mesenteric lymph nodes, the percent of viable immune cells was not different between ZDF and lean rats (average 82 ± 2%).

As a percent of $CD3^+$ lymphocytes, there was a trend (P = 0.09) for ZC and ZS to reduce proportions of helper T-lymphocytes compared to lean rats (67.7 ± 1.1% and 68.0 ± 1.7% vs. 72.8 ± 1.2%, respectively) (Figure 27A, Table 25). There was a trend (P = 0.06) for ZS to reduce the proportion of recent thymic emigrants compared to ZC and lean rats (5.55± 0.75% vs. 7.39 ± 0.32% and 7.76 ± 0.64%, respectively) (Figure 27B, Table 25). ZDF rats fed ZC and ZS had a higher proportion of cytotoxic T-lymphocytes compared to lean rats (28 ± 1.0% and 27.6 ± 1.4% vs. 23.1 ± 1.0%, respectively)(Figure 27C, Table 25). ZDF rats had a reduced CD4 (helper T-lymphocytes) to CD8 (cytotoxic T-lymphocytes) ratio compared to lean rats (Figure 27D). Proportions of total lymphocytes, monocytes/macrophages, and natural killer cells, as a percent of gated cells, T- and B-lymphoctyes as a percent of total lymphocytes, activated or unactivated helper or cytotoxic cells as a percent of $CD3^+$, naive or memory, and recent or late thymic

emigrants, as a percent of helper ($CD3^+$ $CD4^+$) T-lymphocytes, were not different between ZDF and lean rats (Table 25).

T- and B-lymphocyte proportions were also calculated as percent of total lymphocytes (Table 26). ZC and ZS reduced proportions of T-lymphocytes (trend, P = 0.08), helper T-lymphocytes and unactivated helper T-lymphocytes compared to lean rats. ZS reduced proportions of activated helper and tended (P= 0.08) to reduce activated cytotoxic T-lymphocytes compared to lean rats (Table 26). ZS reduced proportions of recent thymic emigrants to a greater extent than MZD (Table 26). ZC and ZS tended (P = 0.09) to lower the CD4 to CD8 ratio compared to lean rats (Table 26). Proportions of cytotoxic and unactivated cytotoxic T-lymphocytes, naïve T-lymphocytes, memory T-lymphocytes, late (mature) thymic emigrants, B-lymphocytes, and immature and mature B-lymphocytes were not different between ZDF and lean rats (Table 26).

Table 24. Spleen and Thymus Weights

	ZDF			
	MZD	**ZC**	**ZS**	**ZC Lean**
Spleen (g)	0.604 ± 0.013^{a}	0.592 ± 0.017^{a}	0.566 ± 0.010^{ab}	0.537 ± 0.013^{b}
Spleen (g/100 g BW)[1]	0.143 ± 0.003^{a}	0.140 ± 0.004^{a}	0.135 ± 0.006^{a}	0.168 ± 0.004^{b}
Thymus (g)[1]	1.07 ± 0.06^{a}	1.07 ± 0.04^{a}	0.89 ± 0.04^{b}	0.54 ± 0.04^{c}
Thymus (g/100 g BW)	0.252 ± 0.014^{a}	0.253 ± 0.010^{a}	0.214 ± 0.010^{b}	0.169 ± 0.011^{c}

Values are means ± SEM, n = 10 except for ZC Lean and ZS where n = 7 and 9, respectively. Superscript letters indicate significant differences ($P < 0.05$) between means. MZD = ZDF marginal zinc deficiency diet, ZC = ZDF zinc control diet, ZS = ZDF zinc supplemented diet, ZC Lean = lean ZDF (+/?) zinc control diet. BW = body weight.

[1]Data were log transformed prior to ANOVA.

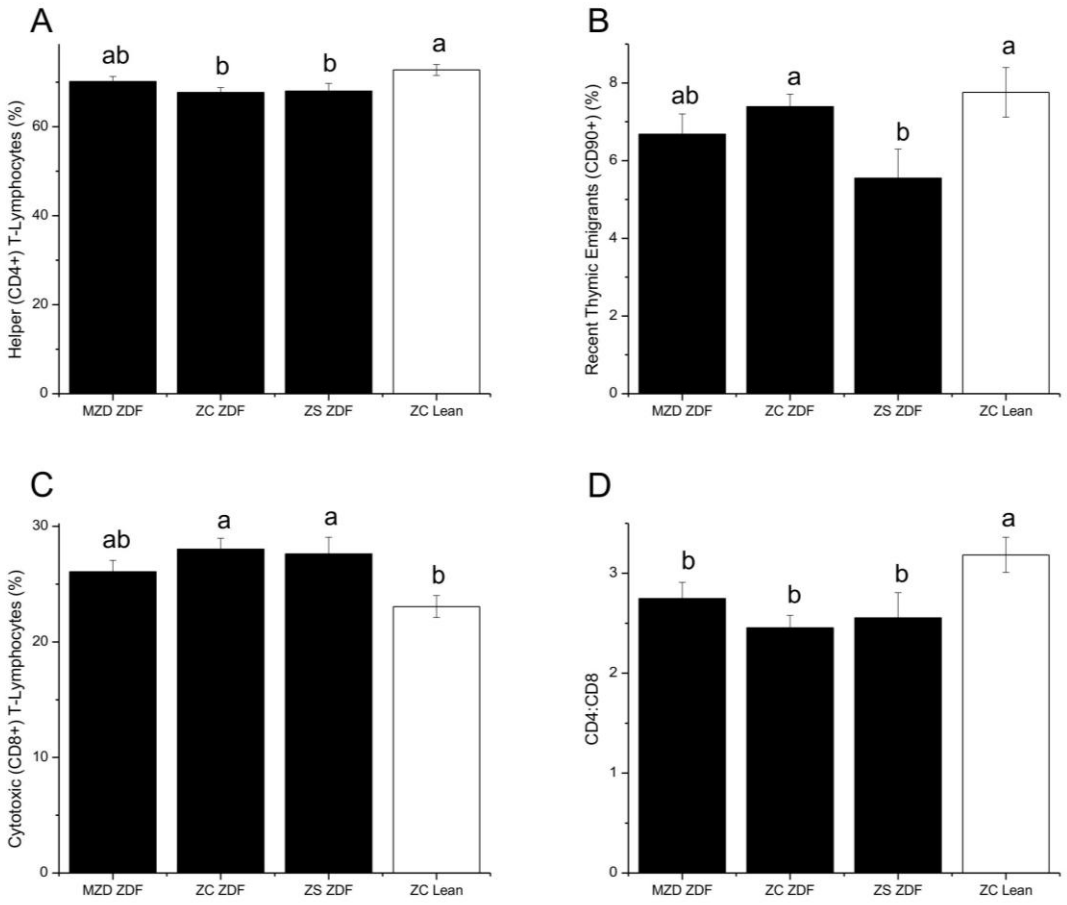


Figure 27. Proportions of helper ($CD3^+$ $CD4^+$) T-lymphocytes (A), recent thymic emigrants ($CD90^+$) (B), cytotoxic ($CD3^+$ $CD8a^+$) T-lymphocytes (C), and helper ($CD4^+$) to cytotoxic ($CD8a^+$) T-lymphocyte ratios (D). Data expressed as means ± SEM, n = 10 except for ZC Lean and ZS where n = 5 and 8, respectively. Different letters indicate significant differences ($P < 0.05$) among means, except for Figure 27A and 27B where different letters indicate a trend for significance at $P = 0.09$ and $P = 0.06$, respectively. Figure 27A, B, and C are expressed as percent of $CD3^+$ T-lymphocytes. Non-parametric testing was used for Figure 27D.

Table 25. Mesenteric Lymph Nodes Immune Cell Proportions

	ZDF			
	MZD	**ZC**	**ZS**	**ZC Lean**
Total Lymphocytes (% of Gated Cells)	64.0 ± 2.2	61.0 ± 2.1	59.2 ± 4.0	68.1 ± 2.0
T-Lymphocytes ($CD3^+$) (% of Total Lymphocytes)	44.4 ± 1.4	41.0 ± 1.5	41.8 ± 1.4	46.1 ± 1.2
Helper T-Lymphocytes ($CD3^+$ $CD4^+$) (% of $CD3^+$)	70.1 ± 1.2[ab]	67.7 ± 1.1[b]	68.0 ± 1.7[b]	72.8 ± 1.2[a]
Activated Helper T-Lymphocytes ($CD3^+$ $CD4^+$ $CD25^+$)	1.10 ± 0.17	1.25 ± 0.18	0.863 ± 0.16	1.26 ± 0.16
Unactivated Helper T-Lymphocytes ($CD3^+$ $CD4^+$ $CD25^-$)	98.9 ± 0.2	98.8 ± 0.2	99.1 ± 0.2	98.7 ± 0.2
Naïve T-Lymphocytes ($CD3^+$ $CD4^+$ $CD45RC^+$)	4.17 ± 0.56	3.47 ± 0.33	3.31 ± 0.68	2.18 ± 0.34
Memory T-Lymphocytes ($CD3^+$ $CD45RC^-$ $CD4^+$)	88.9 ± 1.0	88.8 ± 0.7	91.0 ± 1.5	89.7 ± 0.9
Recent Thymic Emigrants ($CD3^+$ $CD90^+$)	6.68 ± 0.52[ab]	7.39 ± 0.32[a]	5.55 ± 0.75[b]	7.76 ± 0.64[a]
Late (Mature) Thymic Emigrant ($CD3^+$ $CD90^-$ $CD45RC^+$)	75.6 ± 0.9	81.3 ± 8.9	76.4 ± 1.8	79.0 ± 0.9

Cytotoxic T-Lymphocytes ($CD3^+$ $CD8^+$) (% of $CD3^+$)	26.1 ± 1.0^{ab}	28.0 ± 1.0^{a}	27.6 ± 1.4^{a}	23.1 ± 1.0^{b}
Activated Cytotoxic T-Lymphocytes ($CD3^+$ $CD8a^+$ $CD25^+$)	1.14 ± 0.16	1.41 ± 0.16	0.93 ± 0.17	1.44 ± 0.14
Unactivated Cytotoxic T-Lymphocytes ($CD3^+$ $CD8a^+$ $CD25^-$)	98.9 ± 0.2	98.6 ± 0.2	99.1 ± 0.2	98.6 ± 0.1
B-Lymphocytes ($CD45RC^+$) (% of Total Lymphocytes)	51.2 ± 1.9	54.1 ± 2.1	52.4 ± 1.6	49.0 ± 1.5
Immature B-Lymphocyte ($CD90^+$ $CD45RC^+$)	4.64 ± 0.44	4.55 ± 0.30	4.99 ± 0.45	5.56 ± 0.34
Mature B-Lymphocyte ($CD90^-$ $CD45RC^+$)	78.3 ± 2.4	77.1 ± 2.7	75.7 ± 3.6	82.3 ± 0.8
Monocytes/Macrophages ($CD3^-$ $CD4^+$) (% of Gated Cells)	7.13 ± 0.44	6.68 ± 0.49	6.68 ± 0.47	7.76 ± 0.86
Natural Killer Cells ($CD3^-$ $CD8^+$) (% of Gated Cells)	0.94 ± 0.26	0.70 ± 0.16	1.09 ± 0.23	1.08 ± 0.16

Values are means ± SEM, n = 10 except for ZC Lean and ZS where n = 5 and 8, respectively. Different superscript letters indicate significant differences ($P < 0.05$) between means, except for helper T-lymphocytes where $P = 0.09$ and recent thymic emigrants where $P = 0.06$. MZD = ZDF marginal zinc deficiency diet, ZC = ZDF zinc control diet, ZS = ZDF zinc supplemented diet, ZC Lean = lean ZDF (+/?) zinc control diet. Data for lymphocyte subtypes presented as % parent sample, ex. activated and unactivated cytotoxic T-lymphocytes add up to 100% of cytotoxic T-lymphocytes, or all T-lymphocytes plus B-lymphocytes make up 100% of total lymphocytes. Percentages may not add up to exactly 100% due to variance among animals within dietary groups. Refer to Appendix C-F for images of gating.

Table 26. Mesenteric Lymph Nodes T- and B-Lymphocyte Proportions

	ZDF			
	MZD	**ZC**	**ZS**	**ZC Lean**
Total Lymphocytes (% of Gated Cells)	64.0 ± 2.2	61.0 ± 2.1	59.2 ± 4.0	68.1 ± 2.0
T-Lymphocytes ($CD3^+$) (% of Total Lymphocytes)	28.6 ± 1.5^{ab}	25.4 ± 1.5^{b}	24.9 ± 2.1^{b}	31.4 ± 1.1^{a}
Helper T-Lymphocytes ($CD3^+$ $CD4^+$) (% of $CD3^+$)	22.0 ± 1.2^{ab}	17.9 ± 1.7^{b}	17.8 ± 2.1^{b}	24.0 ± 1.4^{a}
Activated Helper T-Lymphocytes ($CD3^+$ $CD4^+$ $CD25^+$)[1]	0.240 ± 0.039^{a}	0.207 ± 0.025^{ab}	0.137 ± 0.016^{b}	0.297 ± 0.028^{a}
Unactivated Helper T-Lymphocytes ($CD3^+$ $CD4^+$ $CD25^-$)	21.8 ± 1.2^{ab}	17.7 ± 1.7^{b}	17.6 ± 2.1^{b}	23.7 ± 1.4^{a}
Naïve T-Lymphocytes ($CD3^+$ $CD4^+$ $CD45RC^+$)	1.05 ± 0.14	0.84 ± 0.10	0.76 ± 0.15	0.65 ± 0.10
Memory T-Lymphocytes ($CD3^+$ $CD4^+$ $CD45RC^-$)	23.0 ± 1.5	21.7 ± 1.6	22.0 ± 1.9	26.9 ± 1.2
Recent Thymic Emigrants ($CD3^+$ $CD90^+$)[2]	1.71 ± 0.19^{b}	1.87 ± 0.09^{ab}	1.27 ± 0.13^{c}	2.31 ± 0.17^{a}
Late (Mature) Thymic Emigrants ($CD3^+$ $CD90^-$ $CD45RC^+$)	19.5 ± 1.3	19.0 ± 1.3	18.5 ± 1.7	23.7 ± 1.0

Cytotoxic T-Lymphocytes ($CD3^+$ $CD8^+$) (% of $CD3^+$)	8.14 ± 0.48	7.35 ± 0.69	6.99 ± 0.63	7.55 ± 0.27
Activated Cytotoxic T-Lymphocytes ($CD3^+$ $CD8a^+$ $CD25^+$)	0.095 ± 0.015^{ab}	0.098 ± 0.012^{ab}	0.060 ± 0.007^{b}	0.108 ± 0.010^{a}
Unactivated Cytotoxic T-Lymphocytes ($CD3^+$ $CD8a^+$ $CD25^-$)	8.05 ± 0.47	7.25 ± 0.68	6.93 ± 0.63	7.44 ± 0.27
CD4:CD8 Ratio[2]	2.75 ± 0.16^{ab}	2.45 ± 0.13^{b}	2.55 ± 0.25^{b}	3.18 ± 0.18^{a}
B-Lymphocytes ($CD45RC^+$) (% of Total Lymphocytes)	31.8 ± 1.3	33.2 ± 1.0	31.4 ± 2.3	32.9 ± 1.4
Immature B-Lymphocytes ($CD90^+$ $CD45RC^+$)	1.45 ± 0.12	1.50 ± 0.10	1.56 ± 0.17	1.84 ± 0.15
Mature B-Lymphocytes $CD90^-$ $CD45RC^+$)	25.0 ± 1.4	25.5 ± 0.9	24.0 ± 2.4	27.1 ± 1.3

Values are means ± SEM, n = 10 except for ZC Lean and ZS where n = 5 and 8, respectively. Different superscript letters indicate significant differences ($P < 0.05$) between means, except for T-lymphocytes and activated cytotoxic T-lymphocytes where $P = 0.08$, and the CD4:CD8 ratio where $P = 0.09$. MZD = ZDF marginal zinc deficiency diet, ZC = ZDF zinc control diet, ZS = ZDF zinc supplemented diet, ZC Lean = lean ZDF (+/?) zinc control diet. Refer to Appendix C-F for images of gating.

[1]Data was log transformed prior to ANOVA.

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

Cell Culture Cytokine Secretion

Mesenteric lymph node cell culture concentrations of the cytokines TNF-α (Table 27), IFN-γ (Table 27), IL-6, and IL-1β (detection limits of 3.72-40000, 26.3-40000, 24.1-40000, 36.9-40000 pg/mL, respectively), were too low to detect in cell culture supernatant at 0 hours of cell culture. At 24 hours of cell culture, TNF-α was detected but there were no differences in supernatant TNF-α concentrations between ZDF and lean rats (Table 26). After 24 hours of stimulation by Concanavalin A in cell culture, TNF-α and IFN-γ cytokines were detected but concentrations were not different between ZDF and lean rats (Table 27).

Table 27. Cell Culture Cytokine Secretion

Cytokine	Time (hours)	ZDF			ZC Lean
		MZD	**ZC**	**ZS**	
TNF-α (pg/mL)	0	ND	ND	ND	ND
	24	19.2 ± 3.1	18.7 ± 2.8	15.3 ± 3.0	13.1 ± 3.1
	24 Concanavalin A Stimulated	149 ± 31	162 ± 26	144 ± 33	131± 42
IFN-γ (pg/mL)	0	ND	ND	ND	ND
	24	ND	ND	ND	ND
	24 Concanavalin A Stimulated	160 ± 96	180 ± 332	179 ± 50	130 ± 54

Values are means ± SEM, n = 10 except for ZC Lean and ZS where n = 7 and 9, respectively. Absence of superscript letters indicates no significant differences ($P > 0.05$) between means. MZD = ZDF marginal zinc deficiency diet, ZC = ZDF zinc control diet, ZS = ZDF zinc supplemented diet, ZC Lean = lean ZDF (+/?) zinc control diet. ND = not detectable.

VI. DISCUSSION

Summary of Main Findings

ZDF rats fed MZD had significantly reduced peri-renal fat pad mass, improved blood lipid levels (elevated HDL-C and reduced triglycerides), and an improvement in the serum urea to creatinine ratio; however, they had pancreatic islet cell hypertrophy and hyperplasia, and reduced β-cell function. ZS had little effect on diabetes, except for elevating urinary glucose excretion and the serum urea to creatinine ratio suggesting reduced renal function. MZD reduced femur, pancreas and urinary zinc concentrations, while ZS elevated femur, pancreas, and serum zinc concentrations. ZS reduced liver copper concentrations but did not alter liver iron concentrations.

Obesity and diabetes elevated serum concentrations of haptoglobin and MCP-1 but reduced adipocyte protein levels of TNF-α and SOCS-3. Therefore, obesity and diabetes enhanced systemic inflammation but lowered local inflammation in adipose tissue. There was a trend for ZS to have lower circulating levels of IFN-γ and IL-6 compared to lean rats indicating impaired cytokine secretion. Although overall, both local and systemic inflammation was not affected by dietary zinc.

In mesenteric lymph nodes, ZC and ZS ZDF rats had increased proportions of cytotoxic ($CD3^+$ $CD8^+$) T-lymphocytes and there was a trend for ZC and ZS to have lower proportions of helper ($CD3^+$ $CD4^+$) T-lymphocytes in ZDF rats compared to lean rats. ZDF rats had a lower CD4 to CD8 ratio compared to lean rats. ZS led to fewer activated helper and cytotoxic T-lymphocytes, and lower proportions of recent thymic emigrants suggesting diminished immune response. Therefore, ZS of 300 mg Zn/kg diet

may be too high since it reduced liver copper concentrations and impaired immune function. In conclusion, MZD seemed to worsen diabetes but had little effect on the immune system, while ZS had little effect on diabetes but may worsen immune function.

Diabetes Parameters

Obesity and Body Composition

ZDF rats are an animal model for obesity and type 2 diabetes because they lack the leptin receptor which impairs appetite suppression, leading to weight gain and obesity (Charles River, 2012). ZDF rats demonstrated hyperphagia by consuming more food per day than lean rats (28 g/d vs. 21 g/d, respectively). ZDF rats were obese since they weighed more than lean rats throughout the study and gained 40% more weight than lean rats by 8 weeks (Table 6). These findings are consistent with the literature (Charles River, 2012; Etgen & Oldham, 2000; Finegood *et al.*, 2001; Sarkozy *et al.*, 2013) where it has been found that ZDF rats weigh more and gain more weight than lean rats. At the end of the study, ZDF rats weighed about 100 g more than lean rats, in agreement with Etgen and Oldham (2000) who found that ZDF rats weighed about 100 g more than lean rats by 12 weeks of age.

As expected, body composition analyses of ZDF rats displayed dramatically greater body fat mass and lower lean body tissue compared to lean rats (Table 7). Higher body fat and lower lean body tissue led to lower total body water since fat tissue contains less water than lean tissue. ZDF rats had greater epididymal, mesenteric, and peri-renal fat pad mass compared to lean rats (Table 8), demonstrating obesity. BMI was more strongly correlated to body fat mass than Lee's index (Figure 3), although total body

water showed a stronger correlation to Lee's Index than to BMI. Therefore, BMI is a more reliable marker of adiposity than Lee's index.

MZD reduced Lee's index, while ZS reduced both Lee's index and BMI, compared to ZC (Table 8). Although, ZDF rats fed ZS had the greatest visceral fat mass (Figure 4). Even though visceral fat mass increased with ZS, the reduction in BMI and Lee's index could be due to a slightly lower total body weight since fat tissue weighs less than lean tissue. Furthermore, excess zinc supplementation may enhance adiposity by activating zinc enzymes which stimulate lipogenesis (Taneja *et al.*, 1996). Also, zinc can stimulate adipogenesis and is involved in adipose tissue metabolism (Coulston & Dandona, 1980; Liuzzi *et al.*, 2005). Therefore, zinc supplementation of 300 mg Zn/kg diet in growing ZDF rats may enhance adiposity. Meanwhile, MZD reduced visceral fat mass without changing feed intake or body weight (Figure 4). The reduction in visceral fat mass but not body weight was due to a reduction in peri-renal fat pad mass. Thus, MZD may reduce and ZS may enhance adipogenesis and adipocyte differentiation. Although, sizing of the adipocytes found no differences among dietary zinc ZDF groups or lean animals (Wu *et al.*, unpublished data).

ZDF rats had 2 cm shorter tail lengths than lean rats (Table 8). The reduction in tail length in ZDF rats has not been previously reported, but has been reported in Zucker (*fa/fa*) rats (Burr, Taylor, & Weiler, 2006) from which ZDF rats were bred. A reduction in tail length could be due to differences in growth related to the *fa/fa* genotype, obesity, and/or diabetes in ZDF rats compared to lean rats.

Overall, ZDF rats exhibited the typical *fa/fa* phenotype by demonstrating hyperphagia and obesity, compared to their lean counterparts.

Glycemic Control and Insulin Resistance

Glucose

ZDF rats were derived from Zucker rats, which were derived from Wistar rats, and have a mutation in the leptin receptor gene along with a mutation which causes spontaneous hyperglycemia (Nugent, Smith, & Jones, 2008). The gene mutation that causes hyperglycemia has not been identified but is likely to be found in the β-cell (Nugent, Smith, & Jones, 2008). ZDF rats have reduced glucokinase expression that results from leptin insensitivity (Wang *et al.*, 1998), leading to altered carbohydrate metabolism. Therefore, lean ZDF rats (+/?) are not supposed to develop hyperglycemia (Wang *et al.*, 1998). Although in the present study, blood glucose concentrations were not different between ZDF and lean rats throughout the 8 week study (Figure 6A). ZDF rats had lower than expected fasting blood glucose concentrations while lean rats had elevated fasting blood glucose levels. Length of fasting, techniques of blood collection and glucose measurement may affect glucose concentrations and may have contributed to abnormal glucose values in the present study.

First, we did not measure non-fasting serum glucose levels, only fasted glucose concentrations. It has been shown that ZDF rats develop non-fasting hyperglycemia at 10 weeks of age (Lee *et al.*, 2010). Charles River (2012) expressed blood glucose values in both the fed and fasted states for ZDF rats, and showed that blood glucose concentrations are higher in the fed than fasted state. Therefore, fasting affects blood glucose levels and

the 5 and 12 hour fasts prior to blood collection would have reduced blood glucose concentrations in ZDF rats compared to the non-fasted state. Although, ZDF rats failed to reach high non-fasting blood glucose levels (about 30 mmol/L by week 12) as Finegood *et al.* (2001) suggests they should.

Another explanation could be the method of blood collection. Finegood *et al.* (2001) and Etgen and Oldham (2000) both used the tail vein for blood draws with the animals in the fed state, then analyzed plasma glucose by enzyme assay methods to quantify glucose concentrations. Both Finegood *et al.* (2001) and Etgen and Oldham (2000) found that ZDF rats were hyperglycemic compared to lean rats. Meanwhile, Sarkozy *et al.* (2013) collected saphenous blood from ZDF rats after a 12 hour fast at weeks 6 and 16 and used a glucometer to analyze serum glucose levels. Sarkozy *et al.* (2013) found that ZDF rats had similar blood glucose concentrations to lean rats at week 6, but at week 16, ZDF rats had higher blood glucose concentrations compared to lean rats, 7.5 vs. 5 mmol/L, respectively. In our study, animals were fasted for 5 hours before the saphenous vein was used for blood collection at weeks 0, 4, and 8, and at the end of week 8 the animals were fasted for 12 hours before euthanization and then the trunk blood was collected. Hyperglycemia increases with longer fasting because there is reduced insulin secretion and greater gluconeogenesis, elevating blood glucose concentrations. The methods used for blood collection in the present study were the same as Dunthorne (2011) used in her Master's Thesis. Therefore, the methods of blood collection should not have affected the results. In the present study, both saphenous (at all time points) and trunk blood showed similar fasting blood glucose concentrations between ZDF and lean rats (Figure 6A and 10A). Consequently, the method of blood

collection and length of fasting do not provide an explanation for the unexpected fasting serum glucose values found.

In the present study, lean rats maintained fairly stable blood glucose levels over the 8 week study which is consistent with the literature (Etgen & Oldham, 2000; Finegood *et al.*, 2001). Finegood *et al.* (2001) and Etgen and Oldham (2000) both found that ZDF rats had only slightly higher blood glucose concentrations than lean rats at baseline (8.5 vs. 6.6 mmol/L at week 6 and 9.38 vs. 7.38 mmol/L at week 7, respectively). In our study we found that lean rats had elevated blood glucose concentrations, similar to that of ZDF rats, throughout the study. At euthanization (after 12 hours of fasting) ZDF rats had blood glucose values of about 17 mmol/L while lean rats had blood glucose values of about 15 mmol/L (Figure 10A). The present results are the first to report that lean ZDF rats developed hyperglycemia. Therefore, the lean rats were hyperglycemic and can only used as a control for obesity but not for type 2 diabetes.

Overall, our data suggests that ZDF rats had borderline low fasting blood glucose values compared to ZDF rats used in other studies (Etgen & Oldham, 2000; Finegood *et al.*, 2001) and our lean rats had higher fasting blood glucose values than lean ZDF rats from another study (Sarkozy *et al.*, 2013). Dietary zinc had no effect on fasting blood glucose concentrations in ZDF rats.

Glucose Concentrations during OGTT

Zinc is involved in glycemic control since it is co-secreted with insulin and acts on α-cells to inhibit glucagon secretion (Ishihara, Maechler, Gjinovci, Herrera, & Wollheim, 2003). During OGTT, there was no difference in glucose concentrations

(Appendix A) or the AUC for glucose between ZDF and lean rats. Even after adjusting for baseline glucose concentrations there was still no difference in glucose tolerance among animal groups (Figure 8A). Peripheral tissues play the predominant role in oral glucose disposal during OGTT (Myllynen, Koivisto, & Nikkila, 1987).This suggests that there was no difference in peripheral tissue glucose uptake among dietary zinc ZDF rat groups or between ZDF and lean rats, although glucose transporters were not measured in peripheral tissues. In contrast, Sarkozy *et al.* (2013) found elevated AUC for glucose during OGTT in ZDF rats compared to lean rats at both 16 and 25 weeks of age, indicative of glucose intolerance.

In summary, both ZDF and lean rats had elevated blood glucose concentrations after a 12 hour fast, and there was no difference in glucose tolerance (even with using an oral dose of 2 g glucose/kg body weight, where most studies use 1 g glucose/kg body weight) between these animals. Dietary zinc had no effect on fasting blood glucose concentrations or glucose tolerance.

Insulin

Insulin resistance and hyperinsulinemia are characteristic of type 2 diabetes, since the pancreas attempts to increase insulin production to maintain normoglycemia (Jansen, Karges, & Rink, 2009; Siwy *et al.*, 2012). Throughout the 8 week study, ZDF rats exhibited hyperinsulinemia compared to lean rats (Figure 6B and 10B) which is in accordance with Charles River (2012) and other studies (Etgen & Oldham, 2000; Lee *et al.*, 2010; Sarkozy *et al.*, 2013; Vora, Zimsen, Houghton, & Anderson, 1996). Obesity stimulates hypersecretion of basal insulin leading to hyperinsulinemia (Milburn *et al.*,

1995), and hyperinsulinemia is a major contributing factor to obesity (Song *et al.*, 2009). Therefore, hyperinsulinemia may be partially responsible for the adiposity found in ZDF rats compared to lean rats.

Zinc is important for insulin and adipocyte interactions (Coulston & Dandona, 1980). Zinc is crucial for insulin signalling since it acts as an insulin mimetic and may enhance the binding of insulin to the insulin receptor (Coulston & Dandona, 1980). In the present study, there was no difference in circulating insulin concentrations among dietary zinc ZDF rat groups. Whereas, the majority of studies suggest that zinc supplementation should improve insulin signalling and sensitivity (Basuki, Hiromura, & Sakurai, 2007; Lemaire *et al.*, 2009; Tang & Shay, 2001). A review by Chausmer (1998) illustrates that zinc and insulin have a physical chemical relationship where zinc controls the maturation, storage and secretion of insulin. Meanwhile, Huber and Gershoff (1973) found that feeding rats high or low zinc diets did not alter pancreatic insulin content and high zinc diets (1200 mg Zn/kg diet) did not affect serum insulin levels. In the present study, dietary zinc did not alter insulin concentrations in ZDF rats but ZDF rats exhibited hyperinsulinemia compared to lean rats. Songergaard *et al.* (2006) suggests that the pancreas can compensate and maintain stable zinc levels during short term low dietary zinc intakes. Therefore, the level of MZD and ZS may not have been adequate to see changes in insulin concentrations or 8 weeks may have been too short to see any differences in insulin secretion.

Insulin Concentrations during OGTT

During OGTT, insulin concentrations (Appendix B) and the AUC for insulin (Figure 9A) were higher in ZDF rats compared to lean rats, but there was no difference in insulin concentrations after adjusting for baseline insulin levels (Figure 8B). Paulsen *et al.* (2010) found comparable insulin concentrations during OGTT (ranging from 1000-1800 pmol/L) in ZDF rats at 14 weeks of age. Although, Paulsen *et al.* (2010) did not adjust for baseline circulating insulin concentrations or compare to lean ZDF rats. In the present study, during OGTT, ZDF rats had higher insulin concentrations to begin with, but there was no difference in glucose-stimulated insulin secretion between lean and ZDF rats. Chronic exposure of β-cells to hyperglycemia leads to β-cell dysfunction and defective insulin secretion (Tanaka, Gleason, Tran, Harmon, & Robertson, 1999). Huber and Gershoff (1973) performed an *in vitro* study of insulin release and found that zinc deficiency reduced glucose-stimulated insulin secretion from pancreata. Additionally, Paulsen *et al.* (2010) demonstrated that after 10 weeks of age, ZDF rats have reduced glucose-stimulated insulin secretion and an AUC for insulin of about 125,000 pmol/L at 14 weeks of age. Furthermore, ZDF rats had an increasingly elevated AUC glucose to insulin ratio over time (Paulsen *et al.*, 2010). In our study, MZD and ZS did not affect glucose-stimulated insulin concentrations and there was no difference in insulin concentrations between ZDF and lean rats after adjusting for baseline concentrations. The AUC for insulin was slightly above the level that Paulsen *et al.* (2010) reported for ZDF rats at 14 weeks of age, and was higher in ZDF rats than lean rats. Although, the AUC ratio for glucose to insulin was lower since the lean rats were hyperglycemic but normoinsulinemic (Figure 9B).

Overall, this data suggests that dietary zinc had little effect on glucose and insulin secretion.

Insulin Resistance and Islet Cell Function

ZDF rats exhibited insulin resistance based on elevated HOMA-IR scores compared to lean rats throughout the 8 week study (Figure 7), an elevated glucose-insulin index (pmol*mmol*min^2) measured during OGTT (Table 10), and a 95% reduction in the insulin sensitivity index (Table 10) in ZDF rats compared to lean rats. These results are in accordance with Sarkozy *et al.* (2013) who found elevated levels of insulin and impaired insulin sensitivity in ZDF rats compared to lean rats.

Beta-cell function was reduced (marked by an increase in HOMA-BCF) in ZDF rats compared to lean rats at 0 and 4 weeks on the study (Figure12). In ZDF rats, zinc deficiency may enhance pancreatic damage (Chausmer, 1998), leading to reduced islet cell function. At 8 weeks of the study, ZC and ZS ZDF rats had HOMA-BCF scores comparable to lean rats, while MZD worsened islet cell function. In addition, HOMA-BCF scores were negatively correlated to pancreas mass (Figure 13).

At euthanization, ZDF rats had reduced absolute and adjusted pancreas mass (Table 11). As type 2 diabetes progresses, a reduction in islet cell mass is common leading to a decline in insulin production (Jansen, Karges, & Rink, 2009). An increase in the amount of fat stored within both the exocrine and endocrine part of the pancreas is related to the development of hyperglycemia in ZDF rats (Lee *et al.*, 2010). In the present study, type 2 diabetes reduced pancreas mass but did not change islet cell area compared to lean rats. The former of these results agree with Finegood *et al.* (2001), who found that

ZDF rats had reduced pancreas weight. Reduced pancreas mass is characteristic of ZDF rats and suggests impaired pancreatic function, and the development of type 2 diabetes (Sarkozy *et al.*, 2013).

At 14 weeks of age, reduced pancreas mass contributed to an impairment in pancreatic function in ZDF rats at euthanization. ZDF rats had substantially elevated HOMA-IR and HOMA-BCF scores compared to lean rats indicating insulin resistance and islet cell dysfunction (Figure 11). Accordingly, ZDF rats developed insulin resistance and islet cell dysfunction in agreement with other studies (Gowda *et al.*, 2013; Sarkozy *et al.*, 2013). As ZDF rats age, plasma glucose concentrations increase while insulin concentrations decrease due to insulin resistance and islet cell dysfunction, meanwhile, lean rats maintain steady glucose and insulin concentrations with age (Etgen & Oldham, 2000). In the present study, HOMA-IR significantly increased in ZDF rats from 0 to 4 weeks and then plateaued from 4 to 8 weeks. HOMA-BCF followed a similar pattern in ZDF rats from 0 to 4 weeks, but declined slightly at week 8, except in MZD where there was a considerable elevation in HOMA-BCF scores at week 8. In ZDF rats, both insulin resistance and islet cell dysfunction tended to plateau over time. Furthermore, MZD exacerbated impaired islet cell function, while ZS had little effect on metabolic and endocrine function in ZDF rats.

Pancreatic islet insulin immunostaining was used to assess islet cell hypertrophy and hyperplasia which indicate an impairment in islet cell function (Figure14 and 15). MZD led to islet cell hypertrophy and hyperplasia, while ZS maintained comparable islet cell size and number to lean rats (Table 11, Figure 14 and 15). Paulsen *et al.* (2010) discovered that in ZDF rats, pancreatic islet cell mass enlarges until 16 weeks of age to

compensate for insulin resistance, and then it declines along with islet cell function. In the present study, MZD substantially increased islet cell mass, which corresponded with a significant elevation in HOMA-BCF scores, suggesting worsened islet cell function compared to ZC ZDF and ZS ZDF rats. Finegood *et al.* (2001) found that ZDF rats have greater islet cell mass compared to lean rats, even though, lean ZDF rats have a 4-fold increase in islet cell mass between 6 to 12 weeks of age to maintain normoglycemia. In the present study, lean rats had small islet cell sizes, similar to ZS and ZC ZDF rats. Therefore, lean rats may have failed to enlarge islet cell mass to compensate for increasing blood glucose levels, contributing to hyperglycemia. Meanwhile, ZS tended to reduce islet cell size and number, with a corresponding reduction in HOMA-BCF, closer to that of lean rats which suggests improved islet cell function.

GPR-39 is a zinc sensing receptor that is expressed in adipose tissue and activated by zinc, thereby activating signal transduction pathways (Egerod *et al.*, 2011; Mlyniec, Budziszewska, Reczynski, Sowa-Kucma, & Nowak, 2013). Epididymal fat GPR-39 protein levels were not different between ZDF and lean rats, although, ZDF rats tended to have reduced levels of GPR-39 (Figure 5, Table 9). GPR-39 deficiency has been associated with islet cell dysfunction, impaired insulin secretion in response to glucose, and glucose intolerance (Holst *et al.*, 2009). Consequently, ZDF rats exhibited hyperinsulinemia, insulin resistance, and islet cell dysfunction, which could be partially due to reduced GPR-39 levels.

Liver Function

Hepatic Steatosis

Hepatic steatosis is common in type 2 diabetes and may precede and contribute to hepatic insulin resistance (Roden, 2006; Schmid *et al.*, 2011). Both absolute and adjusted liver weights were higher in ZDF rats than lean rats suggesting hepatic steatosis (Table 12). ZS reduced absolute liver weight compared to ZC in ZDF rats but after adjusting for body weight, there was no difference in liver weight among dietary zinc groups in ZDF rats. Conversely, Petroulakis (2000) found that at 15 weeks of age, *fa/fa* Zucker rats had greater total and relative liver weights than lean rats, but marginal zinc deficiency (5 mg Zn/kg diet) reduced relative liver weight compared to *fa/fa* Zucker rats fed zinc control (30 mg Zn/kg diet) and to zinc supplemented (150 mg Zn/kg diet) diets. Furthermore, Petroulakis (2000) found that *fa/fa* Zucker rats exhibit hepatic steatosis with 3-fold greater total liver lipid concentrations than lean rats. In agreement with Petroulakis (2000), ZDF rats exhibited hepatic steatosis in the present study with 2-fold larger livers and 2.5 times greater total liver lipid concentrations than lean rats (Figure 16); however there was no effect of dietary zinc levels.

Fasting Serum Lipid Profile

Dyslipidemia, especially elevated triglycerides and low HDL-C, is common in type 2 diabetes (Forbes & Cooper, 2013). Elevated levels of circulating fatty acids can interfere with insulin signalling and enhance hyperinsulinemia, as well as, islet cell hyperplasia (Milburn *et al.*, 1995). In the present study, ZDF rats had similar fasting serum concentrations of free fatty acids compared to lean rats (Figure 17), which is in

congruence with Wendel and Belury (2006). ZDF rats fed ZC had 12-fold higher fasting serum triglyceride levels than lean rats (Figure 17). Wendel and Belury (2006) found similar results with a 4-fold elevation in plasma triglycerides in ZDF rats compared to lean rats. Consequently, elevated free fatty acid and triglyceride levels have been associated with insulin resistance (Wendel & Belury, 2006). MZD elevated fasting serum HDL-C by 1.8-fold and reduced triglycerides by 50% without changing total cholesterol levels (Figure 17). Although MZD reduced triglyceride levels, it did not improve insulin sensitivity or glucose tolerance, as the literature would suggest (Saltiel & Kahn, 2001; Wendel & Belury, 2006). In a clinical trial, zinc supplementation has been found to decrease plasma LDL-C concentrations (Foster & Samman, 2012). Zinc has also been shown to improve total cholesterol levels and the total cholesterol to HDL-C ratio in patients with type 2 diabetes (Gunasekara, Hettiarachchi, Liyanage, & Lekamwasam, 2011). Although, in the present study, fasting serum total cholesterol levels were elevated in ZDF rats and both total cholesterol and LDL-C levels were not affected by dietary zinc (Figure 17).

Renal Function

Urinary Metabolite Excretion

ZDF rats develop diabetes related kidney problems and progressive nephropathy over time (Baynes & Murray, 2009; Suzaki, Ozawa, & Kobori, 2006). Moderate to severe hydronephrosis develops in ZDF rats, and even mild to moderate hydronephrosis appears in lean ZDF rats by week 36 of age (Baynes & Murray, 2009; Vora, Zimsen, Houghton, & Anderson, 1996). Diabetic nephropathy, which leads to renal damage and end-stage

renal disease, is primarily caused by hyperglycemia (Forbes & Cooper, 2013; Suzaki, Ozawa, & Kobori, 2006). Kidneys become hypertrophic with the onset of diabetes (Forbes & Cooper, 2013). In the present study, ZDF rats had greater absolute kidney weights than lean rats, in accordance with Baynes and Murray (2009), although after adjusting for body weight, ZDF rats had similar kidney weights to lean rats (Table 13). This data suggests that absolute kidney weight was higher due to greater body weight and not diabetic hypertrophy.

Urine output has been reported to increase 8-fold by 10 weeks of age and 20-fold by 16 weeks of age in ZDF rats (Baynes & Murray, 2009). In the present study, there was no difference in urinary output at weeks 0, 4 and 8 of the study (age 6 to 14 weeks), between ZDF and lean rats (Table 14, Figure 18). ZC ZDF rats had 2-fold greater urinary glucose excretion compared to lean rats. ZS drastically enhanced urinary glucose excretion by 4.5-fold and 9-fold compared to ZC ZDF rats and lean rats, respectively. Hempe *et al.* (2012) demonstrated that urinary glucose excretion was significantly elevated (about 26 mg/g urine) in ZDF rats (from 12 to 35 weeks of age) compared to lean rats which did not show any urinary glucose excretion. As diabetes progresses, hyperfiltration increases the amount of glucose excreted in the urine (Forbes & Cooper, 2013). In the present study, ZS appears to worsen kidney function and diabetes through increased urinary glucose excretion (Figure 19A).

Urinary urea excretion was elevated in MZD ZDF rats compared to all other groups (Figure 19B), while creatinine excretion (Table 15) remained unchanged between ZDF and lean rats. This is contradictory to Baynes and Murray (2009) who reported decreased urinary creatinine in ZDF rats compared to lean rats which was probably due to

hyperuria. In the present study, ZDF rats did not have hyperuria, and consequently, urine creatinine may not have changed since there was no excessive dilution of the urine. ZDF rats had 2-fold higher urea to creatinine ratios compared to lean rats (Table 15). Therefore, ZDF rats exhibited renal dysfunction through enhanced urinary glucose and urea excretion, and an elevated urea to creatinine ratio.

Serum Markers of Kidney Function

ZS ZDF rats had higher serum urea than MZD ZDF rats and lean rats (Figure 20 B). ZC and ZS ZDF rats had reduced serum creatinine and elevated serum urea to creatinine ratio's compared to lean rats (Figure 20A and C). Creatinine clearance was not different between ZDF and lean rats (Table 15). Similarly, Siwy *et al.* (2012) found that at 2 months of age, creatinine clearance was the same between ZDF and lean rats. Meanwhile, Mega *et al.* (2011) found that ZDF rats had significantly elevated serum urea while serum creatinine remained the same between ZDF and lean rats. Serum creatinine is filtered and not reabsorbed but undergoes tubular secretion, while urea is filtered and not reabsorbed but is not secreted (Lindenfeld & Schrier, 2011). Consequently, levels of blood-urea-nitrogen (BUN) can change while creatinine levels remain fairly constant, providing an indicator of kidney function. An elevated or reduced urea to creatinine ratio is a sign of kidney disorders (prerenal renal dysfunction or intrinsic renal parenchymal disease) and heart failure (Brisco *et al.*, 2013; Lindenfeld & Schrier, 2011). Elevated BUN to creatinine ratios are more often associated with mortality than reduced ratios (Brisco *et al.*, 2013). ZS ZDF rats had an elevated serum urea to creatinine ratio compared to MZD ZDF and lean rats, while MZD improved the urea to creatinine ratio

closer to that of lean rats. This data suggests that ZS worsened while MZD improved kidney function.

Zinc Status

Circulating Zinc Concentrations

Normal zinc homeostasis is maintained through changes in absorption and excretion in the intestine; only during low or high intakes will urinary zinc excretion change (Sandstrom, 1997). Most zinc in the body is bound to proteins, with only small pools of free zinc within cells and the plasma (King, 2011). Plasma concentrations of zinc are tightly regulated by zinc transporters and zinc binding proteins to keep normal plasma levels around 12 to 16 μM (Jansen, Karges, & Rink, 2009). Therefore, serum zinc concentrations may not always reflect cellular zinc status but high intakes of zinc can increase serum zinc levels (Maret & Sandstead, 2006). In the present study, ZDF rats had greater total serum zinc than lean rats (Table 16). ZS elevated serum zinc concentrations and total serum zinc but diabetes and obesity had no effect on serum zinc concentrations (Table 16). It has been reported that marginal zinc deficiency cannot be detected by assessing plasma zinc levels (King, 2011) and that it takes multiple months (>5) for a marginal zinc deficient diet (3-5 mg Zn) to reduce plasma zinc levels in humans (Prasad, 2008). Therefore, serum zinc levels are not a good indicator of zinc status since MZD cannot be detected. Only ZS elicited changes in circulating zinc concentrations among ZDF rats.

Femur Zinc Concentrations

ZS promoted zinc storage in bone, while MZD had the opposite effect on bone zinc concentrations (Figure 21). Femur zinc concentrations were not different between ZC ZDF rats and lean rats indicating no effect of obesity and diabetes on zinc concentrations in bone (Figure 21).

Urinary Zinc Excretion

Diabetes is thought to be associated with hypozincemia through increased urinary zinc excretion or decreased zinc absorption in the gut (Chausmer, 1998). Zinc absorption is slower to respond than zinc excretion to changes in dietary zinc intake (King, 2011). The rate of zinc loss through the urine, skin and intestine is constant and will only change during low or excessive zinc intake (Sandstrom, 1997). In the present study, MZD ZDF rats had reduced urinary zinc excretion to compensate for reduced zinc intake. ZDF rats had higher urinary zinc excretion at week 8 compared to week 0 which is congruent with a review published by Jansen *et al.* (2009), which found that diabetes leads to hyperzincuria. ZC and ZS ZDF rats had 7-fold higher absolute and body weight corrected (µmol/100 g body weight) zinc excretion compared to lean rats and higher creatinine corrected (µg/mg creatinine) zinc excretion compared to both MZD ZDF and lean rats (Table 16). MZD ZDF rats had 4-fold higher absolute and slightly higher creatinine corrected zinc excretion than lean rats even though they were not statistically different. These results agree with a review by Chausmer (1998) that found that both absolute and creatinine corrected urinary zinc excretion are elevated in both type 1 and type 2 diabetes. Chausmer (1998) speculates that hyperglycemia is the underlying cause of hyperzincuria.

Although in the present study, lean rats fed ZC were hyperglycemic yet had lower urinary zinc excretion than ZC ZDF rats. Chausmer (1998) further mentions that the origin of the zinc excreted in the urine is still unknown and hyperexcretion of zinc could be caused from hyperzincuria, or hyperglycemia and hyperinsulinemia, which increase the loss of zinc from tissue stores and plasma. This data suggests that the source of hyperzincuria in diabetes is unknown and that there are more factors involved than hyperglycemia alone. Additionally, serum zinc concentrations were elevated in ZS ZDF rats but not urinary zinc excretion compared to ZC ZDF rats, suggesting that with high dietary zinc intake there is greater storage of zinc in bone to maintain zinc homeostasis.

Pancreatic Zinc Concentrations

Dietary zinc influenced pancreatic zinc concentrations in a dose-dependent manner. Although, in zinc deficiency, the pancreas is able to maintain zinc levels in islets cells, even though total pancreatic zinc content may be reduced (Sondergaard, Stoltenberg, Doering, Flyvbjerg, & Rungby, 2006). Therefore, islet cell zinc concentrations may not have been altered by MZD regardless of any changes to total pancreatic zinc concentrations. ZDF rats fed ZC had lower pancreatic zinc than lean rats fed ZC, indicating a reduction in pancreatic zinc caused by obesity and type 2 diabetes (Figure 22). ZS was able to improve pancreatic zinc concentrations to above the level of lean rats (Figure 22). Therefore, ZS was able to restore any reduction in pancreatic zinc that may have occurred from hyperinsulinemia since zinc is co-secreted with insulin (Ishihara, Maechler, Gjinovci, Herrera, & Wollheim, 2003).

Epididymal Fat Zinc Concentrations

There was a trend for MZD ZDF to have higher epididymal fat zinc concentrations than lean rats (Table 17). This is a novel finding since it has been found that zinc alone can increase glucose uptake in adipocytes; zinc complexes can mimic insulin's action through the insulin signalling pathway and translocation of GLUT 4 from the cytosol to the plasma membrane (Basuki, Hiromura, & Sakurai, 2007; Tang & Shay, 2001; Thirumoorthy *et al*., 2011). Thus, enhanced concentrations of zinc in adipose tissue should promote glucose uptake. But on the contrary, MZD ZDF rats had worsened glucose tolerance even though it wasn't statistically significant. Also, zinc helps to regulate the metabolism of adipose tissue and has the ability to stimulate lipogenesis in adipocytes, even without the presence of insulin (Coulston & Dandona, 1980). Tallman and Taylor (2003) found in C57BL/6J mice, MZD (3 mg Zn/kg diet) and ZS (150 mg Zn/kg diet) had no effect on adipose zinc concentrations, but that high fat fed mice had lower adipose zinc concentrations compared to low fat fed mice. Therefore, it appears that obesity reduces adipocyte zinc transporter expression and potentially, zinc content (Tallman & Taylor, 2003). In the present study, there was no difference in epididymal fat pad mass or epididymal ZnT3 expression among dietary zinc ZDF rat groups. Therefore, differences in adipose zinc concentrations cannot be explained by obesity. Furthermore, MZD ZDF rats had lower femur zinc concentrations suggesting a shift from the storage of zinc in bone to adipose tissue, but the basis for this shift is unknown.

Epididymal Fat Zinc Transporters

There are two main classes of zinc transporters in cells: ZIP, which is responsible for zinc influx into cells, and ZnT, which is responsible for zinc efflux from the cytosol into intracellular organs and from the cytosol into extracellular space (Lemaire *et al.*, 2009). In the present study, protein levels of ZnT3 were not different between ZDF and lean rats (Table 18, Figure 23). ZnT3 is expressed in the greatest amounts in subcutaneous fat from lean humans compared to other fat tissues and obese humans (Smidt *et al.*, 2007). Therefore, epididymal fat may not have been the best type of fat tissue to quantify changes in ZnT3 protein expression.

Liver Zinc Concentrations

Zinc status can affect copper and iron metabolism, bioavailability of other minerals such as folate, and immune function (Sandstrom, 2001). In the present study, obesity and/or type 2 diabetes reduced liver iron, copper and zinc stores in ZDF rats compared to lean rats (Table 19). The literature (Chausmer, 1998; Tallman & Taylor, 2003; Wiernsperger & Rapin, 2010) suggests that zinc status is reduced in diabetes due to hyperglycemia and polyuria. Dietary zinc intake did not affect liver zinc concentrations in ZDF rats but relative liver zinc content was reduced to a greater extend in MZD and ZC fed ZDF rats than ZS ZDF rats (Table 19). Even though ZDF rats had reduced liver zinc concentrations, total liver zinc content was 1.7-fold higher in ZDF rats due to 2-fold higher liver weights than lean rats.

Liver Mineral Concentrations

Micronutrients with chemical similarities can compete for absorption and bioavailability (Sandstrom, 2001). High dose zinc supplementation is associated with copper deficiency because zinc and copper compete for absorption (Plum, Rink, & Haase, 2010). Liver copper concentrations were greatly reduced by ZS, but only slightly reduced by MZD and ZC diets in ZDF rats compared to lean rats (Table 19). Relative liver copper content was only reduced in ZS ZDF rats compared to all other groups (Table 19). ZDF rats had higher total copper content than lean rats, even though total copper content was lower in ZS compared to MZD and ZC ZDF rats (Table 19). Therefore, ZS significantly reduced liver copper concentrations and content due to micronutrient competition; the high dose of dietary zinc depressed copper absorption. High doses (about 120 mg Zn/day) of zinc in humans, in combination with low copper intake, can lead to severe neutropenia and anemia which can be reversed by normalizing zinc intake (Plum, Rink, & Haase, 2010). Consequently, if ZS ZDF rats were fed a ZC diet, liver copper concentrations should return to that of the ZC ZDF rats within a few weeks. In addition, reduced liver copper concentrations suggest that a dietary intake of 300 mg Zn/kg diet of zinc may be too high causing a suppression of copper absorption.

Iron and zinc compete for absorption in a dose-dependent way (Sandstrom, 2001). Liver iron concentrations were lower in ZC and ZS ZDF rats compared to lean rats. Zinc supplementation of 50 mg/d in adult females for 10 weeks reduced iron and copper status (Yadrick, Kenney, & Winterfeldt, 1989). Therefore, higher zinc intakes contribute to lower iron stores in the liver. Reduced iron status may lead to iron deficiency and increased absorption of toxic elements (Sandstrom, 2001). Relative liver iron content was

not different between ZDF and lean rats; meanwhile, total iron content was elevated by 1.7-fold in ZDF compared to lean rats (Table 19). Elevated total iron content is probably due to ZDF rats having double the liver weight as lean rats.

Taken together, this data suggests that the interactions of zinc, copper, and iron, are critical for maintaining mineral balance and health.

Femur Dimensions and Mineral Concentrations

ZDF rats had shorter femurs and narrower femur heads than lean rats (Table 21). ZS reduced total femur length to the greatest extent in ZDF rats, compared to MZD fed ZDF and lean rats (Table 21). This contradicts Hosea et al. (2004) where zinc deficient (<1 mg Zn/kg diet) Sprague Dawley rats had 8% shorter femurs than control rats. In the present study, ZC and ZS fed ZDF rats had smaller hip joint widths than lean rats. Burr *et al.* (2006) determined that femur length, proximal epiphysis width, neck width, and knee width, were smaller in Zucker (*fa/fa*) rats compared to lean rats. Therefore, shorter femur length and smaller head width in ZDF rats could be due to the *fa/fa* genotype. MZD reduced the effect of the *fa/fa* genotype by bringing femur length and width at the hip joint, closer to that of lean rats (Table 21). Although, these changes are not beneficial since reduced femur length and increased width are necessary to support the extra body weight that ZDF rats carry.

Total femur area and bone mineral content were reduced by obesity and type 2 diabetes but bone mineral density was not affected (Table 20). Burr *et al.* (2006) found differing results in *fa/fa* Zucker rats where femur bone area, bone mineral content, and bone mineral density, were not different in *fa/fa* Zucker compared to lean rats. Therefore,

diabetes may be responsible for reducing total femur area and bone mineral content in ZDF rats.

Obesity and diabetes did not alter calcium or phosphorus stores in bone (Table 20). Therefore, ZS enhanced femur zinc storage without changing bone mineral content or density. ZS ZDF rats had shorter femurs, but they did not have reduced total femur area or altered diaphysis width. Taken together, this data suggests that ZS results in a substitution of zinc for other minerals in the bone (disregarding calcium and phosphorus which did not change with dietary zinc intake).

Inflammation

Local Inflammation

ZDF rats are a model of type 2 diabetes with obesity, and therefore, develop associated systemic inflammation (Miranville, Herling, Biemer-Daub, & Voss, 2012). Inflammation is linked to obesity, metabolic syndrome and diabetes (Tataranni & Ortega, 2005). The adipocyte plays a key role in inflammation by secreting cytokines, such as TNF-α and MCP-1. TNF-α and MCP-1 are produced through the activation of multiple signalling cascades involved in obesity and type 2 diabetes, and enhance inflammation (Tataranni & Ortega, 2005). In the present study, adipose tissue protein levels of MCP-1 were not different between ZDF and lean rats, or among dietary zinc groups (Table 22, Figure 24). Meanwhile, Baranowski *et al.* (2012) found that *fa/fa* Zucker rats had almost 6-fold higher adipose tissue MCP-1 protein levels than lean rats. In the present study, adipose tissue levels of both precursor and mature forms TNF-α were about 3-fold higher

in lean rats versus ZDF rats (Table 22, Figure 24). On the contrary, Baranowski *et al.* (2012) reported that *fa/fa* Zucker rats had about 2-fold greater and 10-fold greater adipose tissue protein levels of TNF-α precursor and mature forms, respectively, compared to lean rats. Furthermore, Hotamasligil *et al.* (1993) found that obese adipose tissue secretes double the amount of TNF-α (expressed as the mass of TNF-α secreted per unit of tissue) as lean adipose tissue. Therefore, ZDF rats have altered adipose tissue MCP-1 and TNF-α protein levels compared to Zucker (*fa/fa*) rats from which they were derived. ZDF rats are obese, and consequently, have more adipose tissue than lean rats. Therefore, ZDF rats would have enhanced secretion of TNF-α even though they had low protein levels of TNF-α in adipose tissue. Hotamasligil *et al.* (1993) also found that circulating TNF-α concentrations were higher in obese *db/db* mice compared to lean. Long term exposure to TNF-α in adipocytes can lead to the down regulation of GLUT4, lowered fat and muscle glucose utilization, and peripheral insulin resistance (Hotamisligil, Shargill, & Spiegelman, 1993). Thus, in the present study, the hyperglycemia experienced by the lean rats may have been partially due to high protein expression of TNF-α affecting insulin-stimulated glucose uptake and tissue glucose utilization.

In *db/db* mice, obesity leads to elevated levels of TNF-α which induce SOCS-3 expression in insulin-sensitive tissues, especially adipose tissue (Emanuelli *et al.*, 2001; Kanatani *et al.*, 2007). SOCS-3 is a member of the SOCS family which act as negative feedback mechanisms to reduce cytokine signalling (Emanuelli *et al.*, 2001). In the present study, SOCS-3 protein levels in adipose tissue were elevated in lean rats compared to ZDF rats (Table 22, Figure 24). Obesity has been associated with elevated adipose tissue levels of SOCS-3 contributing to insulin resistance and glucose intolerance

(Kanatani *et al.*, 2007; Palanivel *et al.*, 2012). In *db/db* mice, SOCS-3 is elevated in adipose tissue contributing to insulin resistance and reduced adiponectin production, via inhibition of the JAK/STAT pathway (Kanatani *et al.*, 2007). SOCS-3 can be rapidly induced by insulin and cytokines (especially TNF-α and IL-6), reducing leptin sensitivity and inhibiting insulin signalling through a mediation of the JAK/STAT pathway (Emanuelli *et al.*, 2001; O'Connor, Sherry, Guest, & Freund, 2007; Palanivel *et al.*, 2012).Therefore, lean ZDF rats should have lower SOCS-3 expression than obese, type 2 diabetic, ZDF rats. ZDF rats may have lower SOCS-3 expression due to unknown factors causing a suppression of SOCS-3 or reduced stimulation of SOCS-3. Meanwhile, elevated levels of SOCS-3 in lean rats could be due to enhanced protein levels of TNF-α or elevated circulating cytokines TNF-α, IFN-γ, and IL-6 compared to ZDF rats.

Systemic Inflammation

MCP-1 and haptoglobin are markers of systemic inflammation. MCP-1 attracts monocytes to adipose tissue and they become macrophages. Macrophage infiltration is a key factor in adipose tissue inflammation (Bremer, Devaraj, Afify, & Jialal, 2011; Kolattukudy & Niu, 2012). Elevated haptoglobin levels are an indicator of inflammation, and consequently, haptoglobin levels can be used as an independent marker of coronary vascular disorders in diabetes mellitus (Sadrzadeh & Bozorgmehr, 2004). Both circulating and total serum concentrations of haptoglobin and MCP-1 were elevated in ZDF rats compared to lean rats (Figure 25). MZD tended to reduce serum concentrations of haptoglobin compared to ZC and ZS ZDF rats, although this did not reach statistical significance. These findings are congruent with Miranville *et al.* (2012) who found that ZDF rats had 2.1-fold higher serum concentrations of MCP-1 than lean rats. Miranville *et*

al. (2012) found higher circulating concentrations of haptoglobin in ZDF rats versus their lean controls, although it was not statistically significant. Therefore, ZDF rats exhibit systemic inflammation through elevated circulating haptoglobin and MCP-1 concentrations.

A characteristic of inflammatory disorders is the switch from cellular (Th1) to a less pro-inflammatory humoral (Th2) immune response resulting in an imbalance of cytokines; there is an increase in pro-inflammatory cytokines such as MCP-1 and a down regulation of anti-inflammatory cytokines such as adiponectin (Lu, Xin, Tang, & Shao, 2012; Suganami *et al.*, 2007). Bremer *et al.* (2011) found that individuals with metabolic syndrome have significantly higher subcutaneous adipose tissue levels of MCP-1, CRP, pro-inflammatory cytokines, and an increase in macrophage infiltration, compared to individuals without metabolic syndrome. On the contrary, in the present study, epididymal adipose tissue levels of MCP-1 were not different between ZDF and lean rats, even though circulating concentrations were elevated in ZDF rats compared to lean rats. These data suggest that there was an increase in MCP-1 secretion and/or expression by macrophages and other MCP-1 expressing cells which led to elevated circulating MCP-1 levels in ZDF rats.

Circulating Cytokines

Zinc affects the levels of cytokines though altered gene expression of T-lymphocytes and Th1 and Th2 populations (John *et al.*, 2010; Prasad, 1995). Zinc deficiency has been shown to decrease production of Th1 cytokines, IFN-γ and TNF-α, but does not affect the production of the Th2 cytokine, IL-6 (John *et al.*, 2010). Cytokines

can act as mediators of islet cell death and are associated with the development of diabetes (Egefjord *et al.*, 2009; Marques-Vidal *et al.*, 2012). In the present study, ZDF rats tended to have reduced circulating levels of TNF-α compared to lean rats (Figure 26). This is an interesting finding since it has been reported that ZDF rats have significantly higher circulating TNF-α levels at 8 weeks of age, which consistently increase to 20 weeks of age, while lean rats maintain lower levels throughout this period (Teixeira de Lemos *et al.*, 2009). In addition, elevated levels of TNF-α are found in metabolic disorders such as diabetes (Calle & Fernandez, 2012). Miranville *et al.* (2012) also found that ZDF rats had higher circulating TNF-α, even though it was not statistically significant. In the present study, lower circulating levels of TNF-α could be due to reduced adipose tissue protein expression of TNF-α or reduced secretion.

In both the present study and the study by Miranville *et al.* (2012), ZDF rats had lower levels of circulating IL-6 than lean rats (Figure 26). IL-6 plays an important role in immune function by stimulating T-lymphocyte maturation and both T- and B-lymphocyte activation and differentiation (DiPiro, 1997). IL-6 and TNF-α enhance SOCS-3 expression in adipocytes (Kanatani *et al.*, 2007), and this could explain the higher levels of SOCS-3 expression in adipose tissue of lean rats compared to ZDF rats. ZS reduced circulating IFN-γ and IL-6 concentrations compared to lean rats, but there was no difference between ZC and ZS in ZDF rats (Figure 26). IFN-γ promotes the production of other pro-inflammatory cytokines and enhances cell-mediated immunity by stimulating CD8 cells and macrophages (DiPiro, 1997). Although, reduced secretion of both pro- and anti-inflammatory cytokines in ZS ZDF rats suggests impaired immune function. High doses of zinc may contribute to reduced T-lymphocyte function and an imbalance in

cytokine production (Foster & Samman, 2012). Consequently, the current level of ZS (300 mg Zn/kg diet) may have been too high since it altered cytokine secretion; the reduction in circulating IFN-γ and IL-6 could be due to dysfunctional cytokine signalling and secretion and/or immune response. Meanwhile, MZD did not affect Th1 cytokines, IFN-γ and TNF-α, or Th2 cytokine, IL-6, compared to ZC ZDF rats. Thus, MZD diet at 4 mg Zn/kg diet may not be a low enough level of zinc to produce changes in cytokine secretion.

Total serum TNF-α, IFN-γ, and IL-6 content was not different between ZDF and lean rats but total serum IL-1β was elevated in ZDF rats compared to lean rats (Table 23). Miranville *et al.* (2012) found that ZDF rats had almost 5-fold higher circulating IL-1β than lean ZDF rats. IL-1β is toxic to islet cells and contributes to a down-regulation of zinc transporter expression and insulin gene expression in islet cells (Egefjord *et al.*, 2009; Kutlu *et al.*, 2003). Therefore, elevated total serum IL-1β in ZDF rats could have contributed to the diabetic state expressed in these rats. Pro-inflammatory cytokines such as TNF-α and IFN-γ activate the innate immune system and can cause an impairment of glucose tolerance leading to type 2 diabetes (Tataranni & Ortega, 2005). Lean ZDF rats had higher circulating TNF-α, IL-6, and IFN-γ, and similar circulating IL-1β concentrations compared to ZDF rats, which may have contributed to the abnormal hyperglycemia found in these rats.

Immune Function

Lymphoid Organ Weights

Leptin is required for proper immune responses by controlling the activation of monocytes and natural killer cells, and stimulating the production of cytokines (de Heredia, Gomez-Martinez, & Marcos, 2012). Rodents lacking the leptin receptor or those with a defective leptin receptor (ZDF rats) display reduced T-lymphocyte proliferation and impaired immune response to stimulation (cytokines, mitogens, etc.), as well as lymphoid tissue atrophy (Farooqi *et al.*, 2002; Howard *et al.*, 1999; Lord *et al.*, 1998; Munzberg & Myers, 2005). In the present study, ZDF rats had reduced adjusted spleen weight but higher adjusted thymus weight than lean rats (Table 24). Animal models which are leptin-deficient or have impaired leptin receptors have been reported to have reduced lymphoid organ and immune cell development, and lower cytokine production and secretion (de Heredia, Gomez-Martinez, & Marcos, 2012). Therefore, ZDF rats had lower adjusted spleen weight and reduced circulating cytokines TNF-α, IL-6, and IFN-γ, than lean rats. ZS tended to reduce absolute spleen weight closer to that of lean rats compared to MZD and ZC ZDF rats (Table 24). ZS led to thymic atrophy by reducing both absolute and adjusted thymus weights compared to MZD and ZC ZDF rats (Table 24). On the contrary, a review by Blewett and Taylor (2012) found that zinc deficiency reduced lymphoid organ weight (ex. spleen and thymus) in rodents. In addition, Hosea *et al.* (2004) found that zinc deficiency in a growing rat model significantly reduced absolute spleen and thymus weight but there were no changes in absolute numbers of T-lymphocytes indicating no lymphopenia. Therefore, there was no reduction in lymphoid

organ weight in MZD ZDF rats since MZD does not cause severe/overt zinc deficiency. Meanwhile, high dose zinc supplementation in humans (300 mg Zn/d or 20 times the recommended daily allowance) may cause immunosuppression and decreased lymphocyte proliferation (Shankar & Prasad, 1998). Malnutrition can result in severe thymic atrophy (thymocyte apoptosis and reduced cell proliferation) and immunodeficiency (Savino & Dardenne, 2010). Therefore, malnutrition in the form of both deficiency and toxicity of vitamins and trace elements (ex. zinc) can lead to thymic atrophy. In the present study, the level of zinc in the ZS diet may have been too high, causing toxicity, since it led to lymphoid atrophy by reducing both absolute and adjusted thymus weight.

Immune Cell Proportions

Zinc is required for the physiological functioning of the both the innate and adaptive immune systems, and especially for the development and maturation of T-lymphocytes (Jansen, Karges, & Rink, 2009). The innate immune system involves natural killer cells and monocytes/macrophages (John *et al.*, 2010). The adaptive immune response involves both T- and B-lymphocytes; B-lymphocytes induce humoral immunity and produce antibodies, while T-lymphocytes induce cell-mediated immunity using helper ($CD4^+$) and cytotoxic ($CD8^-$) cells (John *et al.*, 2010). Helper T-lymphocytes are classified into 2 groups: Th1 cells which produce IFN-γ, IL-2, and TNF-α, and enhance cell-mediated immunity, and Th2 cells which produce IL-4, IL-6, and IL-10, and enhance humoral immunity (DiPiro, 1997; John *et al.*, 2010). The mechanisms of zinc's involvement in the immune system are still unknown but may involve: zinc is essential

for over 300 enzymes and for DNA and RNA stabilization, which may play a role in lymphocyte proliferation, zinc is required for thymulin which is necessary for T-lymphocyte maturation, cytotoxicity and cytokine secretion, and zinc contributes to membrane stabilization and plays an important role in the regulation of apoptosis within lymphocytes (Dardenne, 2002).

In the present study, immune cell proportions were altered by dietary zinc intake. Both ZC and ZS ZDF rats had increased proportions (expressed as percent of $CD3^+$ cells) of cytotoxic T-lymphocytes and reduced proportions of helper T-lymphocytes compared to lean rats (Figure 27). Meanwhile, MZD in ZDF rats brought proportions (expressed as percent of $CD3^+$ cells) of cytotoxic and helper T-lymphocytes closer to that of lean rats than ZC and ZS (Figure 27). Although, ZC and ZS altered proportions (expressed as percent of $CD3^+$ cells) of cytotoxic and helper T-lymphocytes in ZDF rats, the CD4:CD8 ratio did not change; the CD4:CD8 ratio was lower in ZDF rats than lean rats (Figure 27). ZDF rats lack the leptin receptor, and consequently, may exhibit defective immune function (de Heredia, Gomez-Martinez, & Marcos, 2012). Thus, ZDF rats had a lower CD4:CD8 ratio which suggests impaired immune function and could explain the reduced levels of circulating cytokines TNF-α, IL-6, and IFN-γ, compared to lean rats.

Furthermore, the innate immune system plays a significant role in the development of type 2 diabetes. ZDF rats fed ZC had reduced proportions (expressed as percent of lymphocytes) of T-lymphocytes and helper T-lymphocytes compared to lean rats (Table 26). In addition, both ZC and ZS tended to lower the CD4 to CD8 ratio (expressed as percent of lymphocytes) in ZDF rats compared to lean rats (Table 26), suggestive of impaired immune function. ZS reduced proportions (expressed as percent

of lymphocytes) of activated helper and activated cytotoxic T-lymphocytes, and recent thymic emigrants to the greatest extent compared to lean rats (Table 26), indicating worsened immune function. In addition, ZS may contribute to an under-activation of immune cells and a diminished immune response since it reduced the proportions (expressed as percent of lymphocytes) of activated helper and cytotoxic T-lymphocytes. Meanwhile, MZD had the least effect on immune cell proportions in ZDF rats since it only reduced proportions (expressed as percent of lymphocytes) of recent thymic emigrants compared to lean rats.

Zinc deficiency is known to adversely affect the innate and adaptive immune systems, especially lymphocyte proliferation and T-lymphocyte function (John *et al.*, 2010). Although, in the present study, MZD ZDF rats had the least changes in immune cell proportions versus ZC or ZS ZDF rats, when compared to lean rats. MZD may have had limited effects on CD4 and CD8 cell proportions since proportions of recent thymic emigrants were not altered and mature T-lymphocytes are more resistant to zinc deficiency (John *et al.*, 2010). Sazawal *et al.* (1997) found that zinc supplementation of 10 mg per day (20 mg during diarrhea) in children improved cellular immunity by increasing T-lymphocytes, helper T-lymphocytes, and the CD4:CD8 ratio. Thus, the data from the present study suggests that MZD may improve proportions of helper and cytotoxic T-lymphocytes in ZDF rats with type 2 diabetes.

Diabetes-prone BioBreeding rats have reduced thymocyte export and recent thymic emigrants due to apoptosis, predisposing them for autoimmunity (Iwakoshi *et al.*, 1998). In the present study, ZS reduced proportions of recent thymic emigrants compared ZC ZDF and ZC lean rats. This suggests that ZS exacerbated diabetes by reducing recent

thymic emigrants and by possibly making them more susceptible to apoptosis. ZS has been reported to impair lymphocyte proliferation and T-lymphocyte response (Chandra, 1984) and can induce immunosuppression (Dardenne, 2002). Therefore in the present study, ZS of 300 mg Zn/kg diet may promote immunosuppression and a state of immune dysfunction by reducing proportions of developing T-lymphocytes (recent thymic emigrants), and activated helper and cytotoxic T-lymphocytes. Although, proportions (expressed as both percent of $CD3^+$ cells and percent of lymphocytes) of total lymphocytes, unactivated cytotoxic T-lymphocytes, naïve T-lymphocytes, memory T-lymphocytes, late (mature) thymic emigrants, B-lymphocytes, immature and mature B-lymphocytes, and proportions (expressed as percent of $CD3^+$ cells) of T-lymphocytes, activated and unactivated helper T-lymphocytes, monocytes/macrophages, and natural killer cells, and proportions (expressed as percent of lymphocytes) of cytotoxic T-lymphocytes, were not different among dietary zinc groups or between ZDF and lean rats (Table 25 and 26).

Cell Culture Cytokine Secretion

Cell culture and stimulation (Concanavalin A) of the mesenteric lymph node immune cells did not show any differences in cytokine secretion among dietary zinc groups or between ZDF and lean rats (Table 27). There were vast differences in cytokine concentrations within experimental groups, leading to a large variance and insignificant results. The concentrations of all cytokines (TNF-α, IFN-γ, IL-1β, and IL-6) were below detectable levels at 0 hours of cell culture (unstimulated). At 24 hours of unstimulated cell culture, only TNF-α was detected, and at 24 hours of stimulated cell culture both

TNF-α and IFN-γ were detectable but not IL-1β or IL-6. Therefore, there were no detectable differences in both unstimulated and stimulated cytokine secretion among dietary zinc groups or between ZDF and lean rats. This is a novel finding since the immune cells from mesenteric lymph nodes of ZDF rats have not been previously studied. Mitogen-stimulated cytokine secretion can be used as an assessment of immune function (ex. how well cells respond to an immune challenge). Baranowski *et al.* (2012) did not find differences in Concanavalin A (2.5 mg/L) stimulated splenocyte concentrations of IL-2, IL-6, TNF-α, or IFN-γ, after 48 hours of cell culture, between *fa/fa* and lean Zucker rats. Although, Baranowski *et al.* (2012) reported a slight reduction in both unstimulated and stimulated splenocyte IL-10 secretion (1000 and 800 pg/mL vs. 900 and 600 pg/mL) in fa/fa Zucker rats compared to lean rats. Consequently, the data from the present study suggests that immune cells from the mesenteric lymph nodes of ZDF rats have alter cytokine secretion, secrete much lower concentrations of cytokines, or that the cells should have been stimulated for 48 hours to produce better results.

VII. SUMMARY

Diabetes Parameters

ZDF rats displayed all of the typical parameters of obesity and type 2 diabetes including hyperglycemia, hyperinsuinemia, insulin resistance, hepatic steatosis, and hyperlipidemia. Lean ZDF rats exhibited abnormal hyperglycemia which could be due to a variety of genetic and/or metabolic impairments and factors. MZD significantly reduced peri-renal fat pad mass, improved blood lipid levels (increased high-density lipoprotein cholesterol and decreased triglycerides), and improved the serum urea to creatinine ratio; however, it led to pancreatic islet cell hypertrophy and hyperplasia, and reduced β-cell function. Thus, MZD primarily affected diabetes parameters by impairing pancreatic function. Meanwhile, ZS had little effect on diabetes, except for elevating urinary glucose excretion and the serum urea to creatinine ratio suggesting impaired renal function. MZD reduced urinary zinc excretion to compensate for reduced dietary intake, while ZS had elevated serum zinc concentrations which are usually kept within a narrow range. Bone and pancreas zinc concentrations fluctuated according to dietary zinc intake and availability. ZS reduced liver copper concentrations and may result in a substitution of zinc for other minerals in the bone (disregarding calcium and phosphorus).

A review by Beletate *et al.* (2007) assessed the ability for zinc supplementation to prevent type 2 diabetes in adults. They found that there were no differences in anthropometric measures, blood glucose, insulin, leptin and zinc, lipid metabolism, urinary zinc excretion, and insulin resistance, between zinc supplemented and placebo groups (Beletate, El Dib, & Atallah, 2007). The present study found similar results where

MZD may worsen diabetes but ZS had little effect on metabolic and endocrine function in ZDF rats. Therefore, zinc supplementation may not be beneficial for preventing the development of type 2 diabetes.

Inflammation

In summary, ZDF rats had elevated circulating concentrations of haptoglobin and MCP-1, and reduced adipose tissue protein levels of TNF-α and SOCS-3; obesity and diabetes enhanced systemic inflammation but reduced local inflammation in adipose tissue. Lean rats had higher circulating levels of TNF-α, and higher IL-6, and IFN-γ (even though it was not statistically significant), and similar concentrations of IL-1β, compared to ZDF rats, while ZS in ZDF rats lowered circulating IFN-γ and IL-6 concentrations compared to lean rats. Overall, obesity and type 2 diabetes had a greater effect than dietary zinc on both local and systemic inflammation.

Immune Function

ZDF rats had reduced adjusted spleen and higher adjusted thymus weights than lean rats. ZS enhanced thymic atrophy compared to MZD and ZC in ZDF rats. ZDF rats fed ZC had reduced proportions of T-lymphocytes and helper T-lymphocytes in mesenteric lymph nodes compared to lean rats suggesting altered immune function. ZC and ZS reduced proportions of helper T-lymphocytes and elevated cytotoxic T-lymphocytes without changing the CD4:CD8 ratio, however the CD4:CD8 ratio was lower in ZDF rats compared to lean rats. ZS reduced proportions of activated helper and cytotoxic T-lymphocytes compared to lean rats, and reduced proportions of recent thymic

emigrants compared to ZC ZDF and lean rats. Therefore, ZS negatively altered immune cell proportions with the potential for worsened immune function. Furthermore, ZS may diminish the immune response due to an under-activation of helper and cytotoxic T-lymphocytes. Meanwhile, MZD had the least effect on immune cell proportions in ZDF rats since it only resulted in reduced proportions (expressed as percent of lymphocytes) of recent thymic emigrants compared to lean rats.

There was no difference in both unstimulated and stimulated immune cell cytokine secretion between ZDF and lean rats. Overall, ZDF rats have an imbalance in helper and cytotoxic T-lymphocytes which may result in immune dysfunction. Furthermore, ZS may exacerbate changes in immune cell proportions in ZDF rats.

Overall Summary

Zinc plays an important role in diabetes and affects many aspects of diabetes, including glycemic control. Many of the symptoms of zinc deficiency and diabetes are the same and are related to an increase in inflammation. Immune dysfunction is commonly associated with diabetes and can be influenced by zinc status. Although, the relationship among zinc status, type 2 diabetes, and immune dysfunction is not clear. The present study attempted to determine the effects of zinc status on type 2 diabetes and the immune system in ZDF rats. The results found that MZD may exacerbate diabetes but had little effect on the immune system, while ZS had little effect on diabetes but may worsen immune function. Dietary zinc had little effect on both local and systemic inflammation. The results indicate that even marginal zinc deficiency can have a large negative impact on the progression of diabetes. The level of zinc supplementation used in

this study may have been too high for optimal improvements in diabetes, inflammation, and immune function. Therefore, it is the balance between zinc deficiency and toxicity that is necessary for optimal health improvements. Further investigations using lower levels of zinc supplementation are needed to determine the ability of zinc to improve diabetes and immune system function.

VIII. STRENGTHS

- This study was well designed since it used the best animal model, the ZDF rat, for testing the hypotheses.
- Multiple controls (pair-weighed animals, lean control, and ZDF control) were used to ensure changes related to zinc intake were independent of other factors.
- The use of a semi-purified diet allowed for the modification of the levels of zinc in the diet without altering macronutrient composition or other micronutrients.
- Marginal zinc deficiency was used instead of severe zinc deficiency since subclinical zinc deficiency is more common in the general population than overt zinc deficiency. The level of marginal zinc deficiency was at an appropriate level to avoid weight loss or clinical signs of zinc deficiency, but led to 50% lower femur zinc concentrations than those of both ZDF and lean rats fed ZC.
- This study was able to assess the effects of dietary zinc intake on multiple systems within the body, from a whole body to a molecular scale.
- To the best of our knowledge, this is the first study to assess the effects of zinc on both diabetes and the immune system in a diabetes model. Therefore, it helps to bridge the knowledge gap between dietary zinc intake and the parameters of diabetes as well as, associated inflammation and immune dysfunction.

IX. LIMITATIONS

- The major limitation to this study was the lean control ZDF rats developing hyperglycemia after an overnight fast and ZDF rats did not attain the expected hyperglycemia. The lean control rats are not supposed to develop hyperglycemia and therefore should have been a proper control for both obesity and type 2 diabetes. As a result, the lean rats could only be used to control for obesity and not type 2 diabetes. Therefore, any changes that occurred between ZDF and lean rats can only be explained independently of obesity and are associated with diabetes. Although, we were able to compare among dietary ZDF rat groups to assess the effects of varying dietary zinc intakes on the parameters of diabetes, inflammation, and immune function. The amount of fat used in the diet was the same amount and as the recommended Purina # 5008 diet. Although the source of fat used in the present study was soybean oil whereas the Purina # 5008 contains porcine animal fat. Therefore, the type of fat may have played a role in the under-development of hyperglycemia in ZDF rats.
- Each animal group had a sample size of 10 animals to begin with, but due to unforeseeable factors, only 7 lean rats remained at the end of the 8 week study. The reduction in animals created uneven sample sizes among experimental groups and an unbalanced ANOVA. Therefore, the results may be skewed slightly and should be considered with caution.
- This study was 8 weeks long which may not have been long enough to see changes in glucose tolerance, inflammation, and other parameters, among dietary zinc

groups. Therefore, a longer study may have led to enhanced changes and more significant results.

- The high dose of zinc used for ZS in the present study was not reflective of dietary intake in the human population; we used a dose that was 10-fold greater than the recommended daily intake for rats, whereas the human upper limit is 5-fold the recommended daily allowance. Also, our level of ZS was a pharmacological dose that would only be achievable in the human population through supplementation, not diet.
- Recent thymic emigrants (expressing $CD90^{+}$) are unique to the rat species so our finding that ZS reduced recent thymic emigrants is difficult to translate to humans, but there are other markers to identify recent thymic emigrants in humans.
- Mitogen-stimulated mesenteric lymph node immune cells failed to produce detectable levels of cytokines or significant results. Perhaps a longer incubation time in cell culture (ex. 48 hours) or a higher level of Concanavalin A (or other monocyte/macrophage mitogens) would have produced detectable levels of cytokines.

X. CONCLUSIONS

ZDF rats exhibit all of the typical parameters of obesity and diabetes such as hyperglycemia, hyperinsulinemia, insulin resistance, hyperlipidemia, hepatic steatosis, and inflammation, which makes them the best animal model for this study. Lean ZDF rats were hyperglycemic and it can be speculated that the abnormal hyperglycemia was due to genetic factors, inflammation, free fatty acid lipotoxicity of beta-cells, altered response to hyperglycemia, and/or other unknown factors. Therefore, the lean rats were used as a control for obesity but not for type 2 diabetes.

MZD may improve obesity and the blood lipid profile, but worsens diabetes by impairing pancreatic and beta-cell function. ZS had little effect on the parameters of diabetes, other than impairing renal function. Obesity and type 2 diabetes had a greater effect than dietary zinc on inflammation. Lean rats had higher levels of circulating cytokines TNF-α, IL-6, and IFN-γ, and adipose tissue TNF-α and SOCS-3, although, they had lower circulating haptoglobin and MCP-1 concentrations than ZDF rats. Therefore, ZDF rats had altered adipocyte protein levels of cytokines and cytokine secretion. Immune function may be impaired in ZDF rats due to altered T-lymphocyte immune cell proportions, compared to lean rats. In ZDF rats, ZS reduced proportions of recent thymic emigrants and may diminish the immune response due to an under-activation of helper and cytotoxic T-lymphocytes. Furthermore, mesenteric lymph node immune cell proportions were altered by obesity, and could be due to changes in adipocyte cytokine protein levels, circulating cytokines and inflammatory markers. ZS appeared to exacerbate while MZD ameliorated obesity related changes in mesenteric lymph node immune cell proportions. Thus, ZS of 300 mg Zn/kg diet may have been too high since it

worsened kidney function, reduced liver copper concentrations, and impaired the immune system by altering cytokine secretion and immune cell proportions. In conclusion, it is the balance between marginal zinc deficiency and toxicity that is necessary to improve the parameters of diabetes and maintain proper immune function.

XI. FUTURE DIRECTIONS

- This study should be repeated using an extra control for type 2 diabetes, in case lean ZDF rats developed hyperglycemia again. Controls animals that could be used are obese control Zucker (*fa/fa*) or non-obese control Wistar rats, from which ZDF rats were derived; both of these animal models do not develop hyperglycemia on a normal AIN-93G diet. Therefore, changes among animal groups could be explained independently of diabetes.
- The level of ZS used in this study appeared to negatively impact the immune system without having many affects on the parameters of diabetes. Thus, future experiments should reduce the level of ZS to eliminate the negative consequences and to allow the research to be extrapolated to humans (within the upper limit of daily intake for zinc). Although, Petroulakis (2000) showed little effect of 150 mg Zn/kg diet in Zucker (*fa/fa*) rats.
- Future experiments could use a cross-over design to see if ZS can reverse the effects of MZD on diabetes, especially the pancreas, in ZDF rats.
- Additional markers of inflammation, such as macrophage infiltration in adipose tissue, are needed to gain a better understanding of how zinc influences inflammation.
- Levels of thymulin could be measured to help explain the differences in thymus weight and T-lymphocyte populations that were discovered among animal groups.
- Mitogen-stimulated mesenteric lymph node immune cells could be repeated with a higher dose of Concanavalin A or an enzyme-linked immunosorbent assay (ELISA, with a lower detection limit [MSD = 2.5 standard deviations above 0,

ELISA = 2 standard deviations above 0]) could have been used to evaluate changes in immune response and T-lymphocyte cytokine secretion.

XII. IMPLICATIONS

No studies to date have concurrently assessed the effects of zinc on diabetes and immune function. The results of this study help generate a better understanding of the role of zinc in diabetes and its associated complications such as cardiovascular disease and immune dysfunction. Zinc deficiency is common in people with diabetes, and these results help characterize the changes that occur within the body. It was found that the pancreas is the most sensitive tissue to zinc status and diabetes; even marginal zinc deficiency impaired pancreatic function. Consequently, this study helped to uncover the subclinical signs of marginal zinc deficiency so that early detection and diagnosis can be used to help prevent zinc deficiency and its associated complications.

Immune system dysfunction plays a key role in development and exacerbation of the signs of diabetes. Diabetes and immune dysfunction are interrelated; diabetes contributes to immune dysfunction, and immune dysfunction worsens diabetes. Therefore, this study helped to uncover the relationship between diabetes and the immune system, which are both influenced by zinc status. This study demonstrated that high dietary intake of zinc may have negative consequences for the immune system. Thus, caution should be used when prescribing zinc supplementation to humans with type 2 diabetes in order to avoid adverse outcomes.

Overall, the results of this study help to describe the relationship and mechanisms behind nutrition and disease outcomes, and expand the knowledge of zinc's role in diabetes and immune function.

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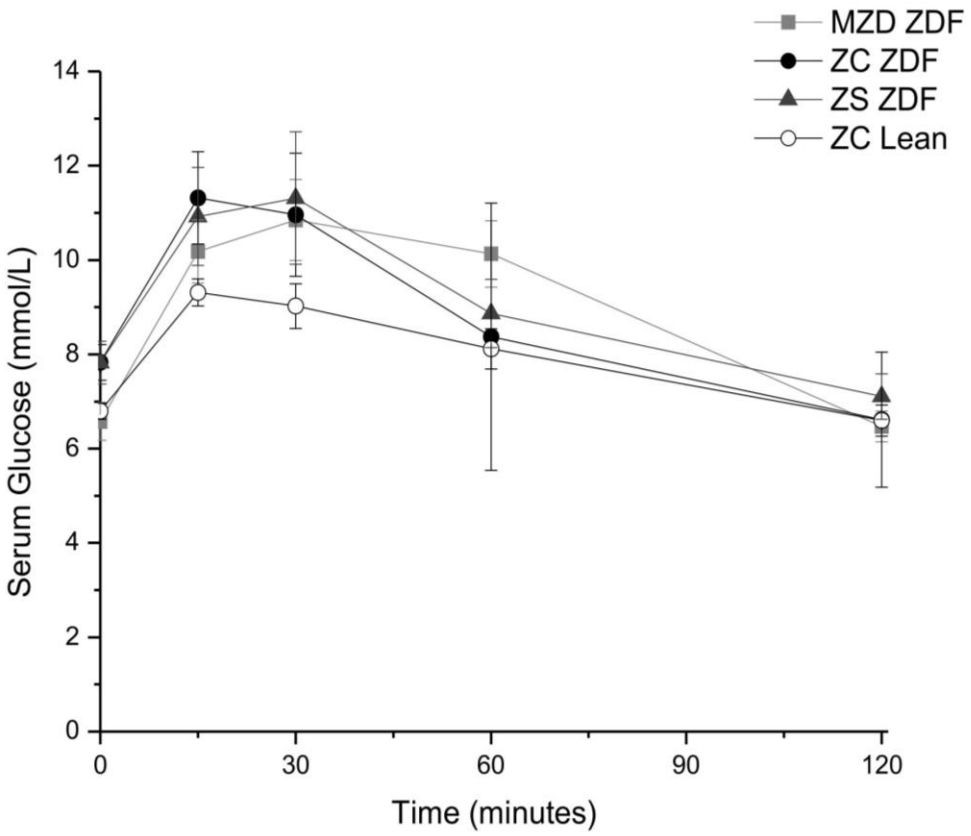
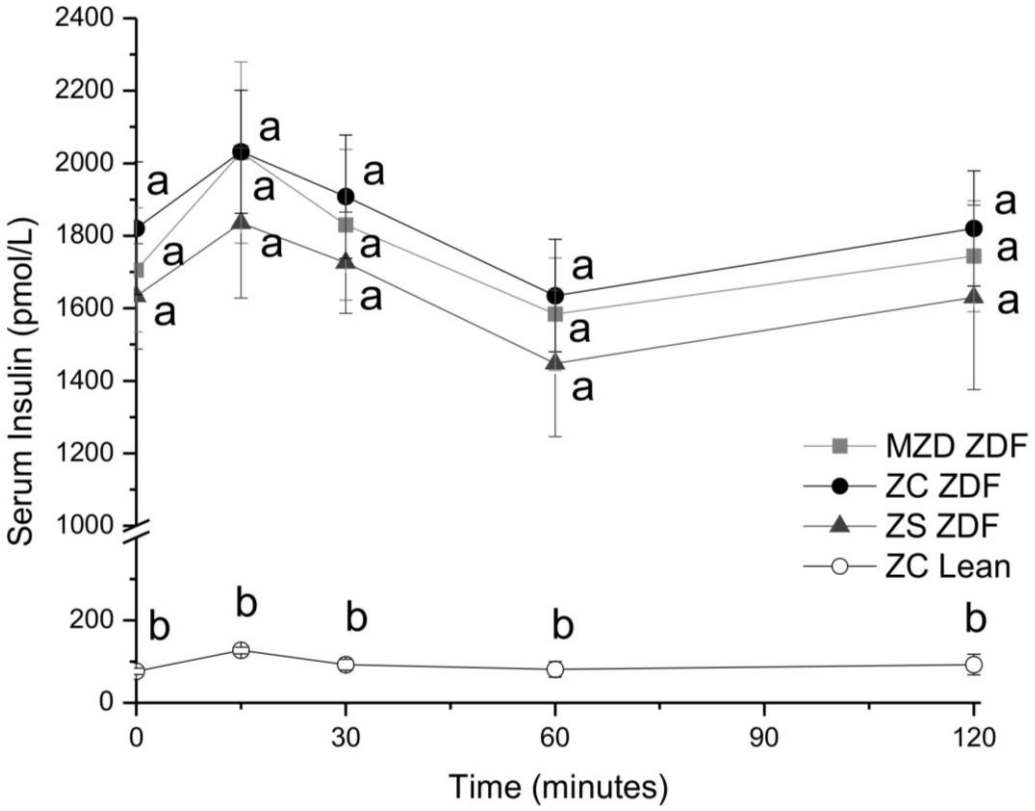
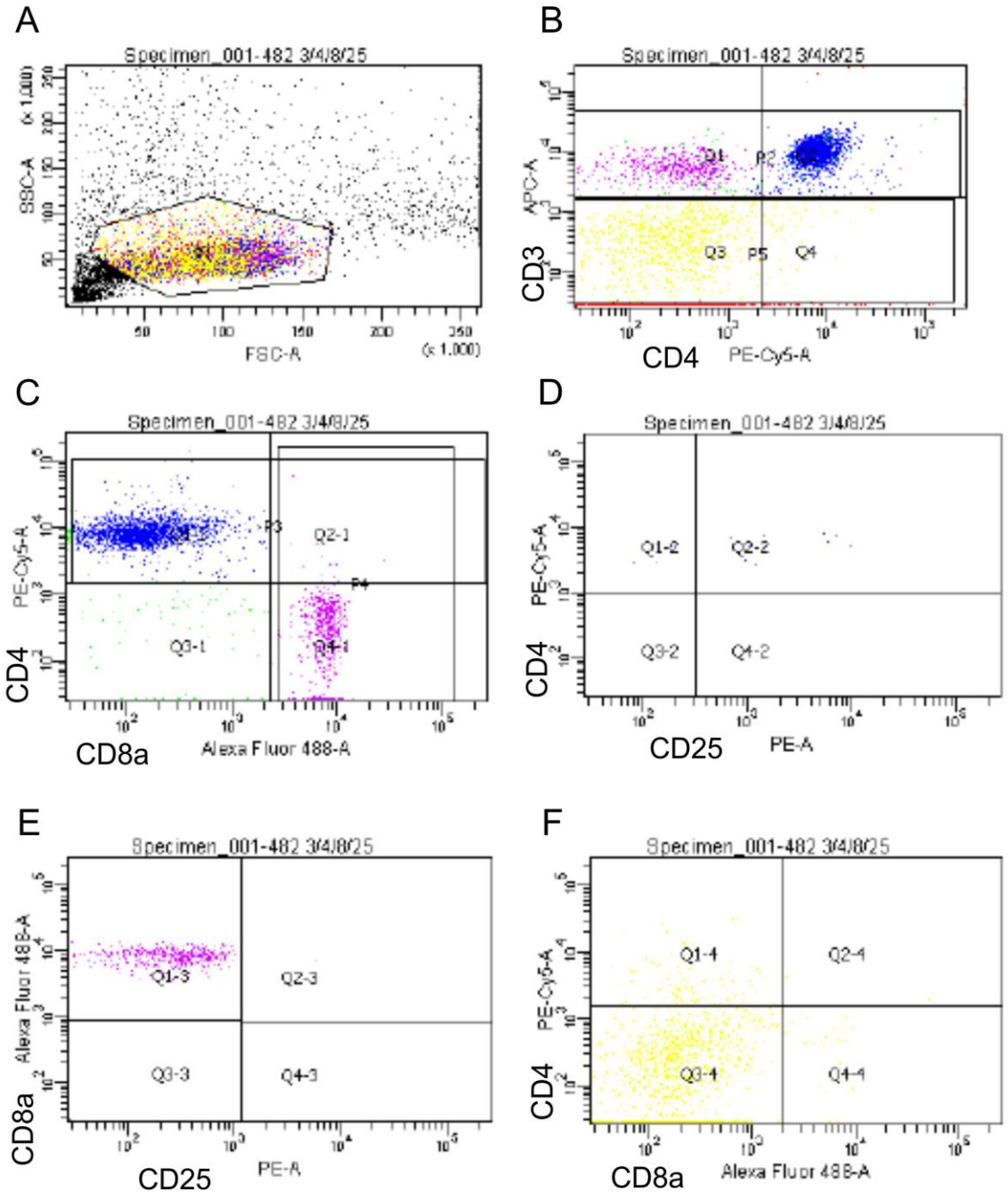
XIV. APPENDICES



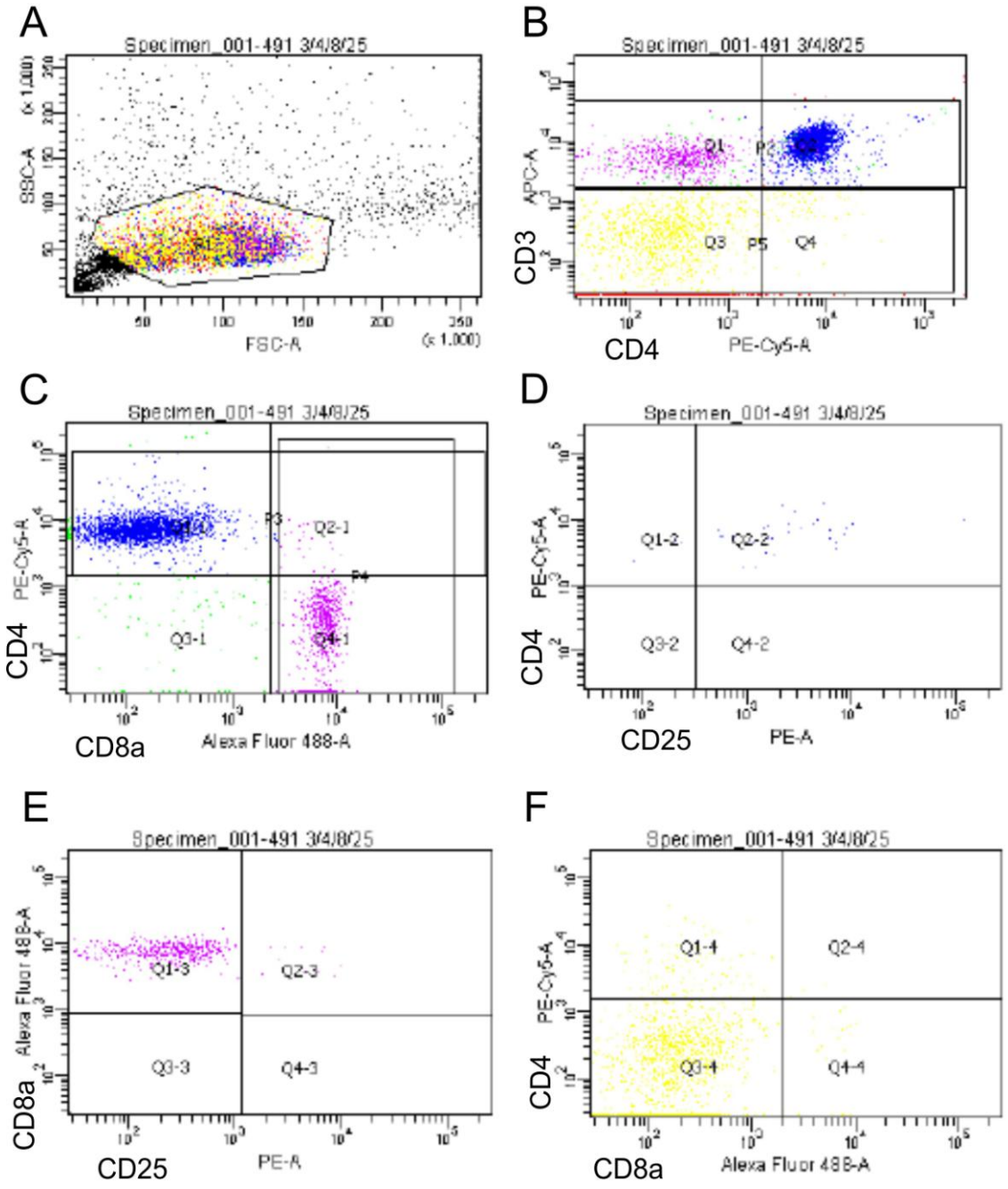
Appendix A. Absolute OGTT serum glucose concentrations. Data expressed as means ± SEM, n = 10 except for ZC Lean and ZS where n = 7 and 9, respectively. There were significant main effects of time but not time x diet interaction for serum glucose.



Appendix B. Absolute OGTT serum insulin concentrations. Data expressed as means ± SEM, n = 10 except for ZC Lean and ZS where n = 7 and 9, respectively. There were significant main effects of time and time x diet interaction for serum insulin. Different letters indicate significant differences ($P < 0.05$) among means at the same time point.

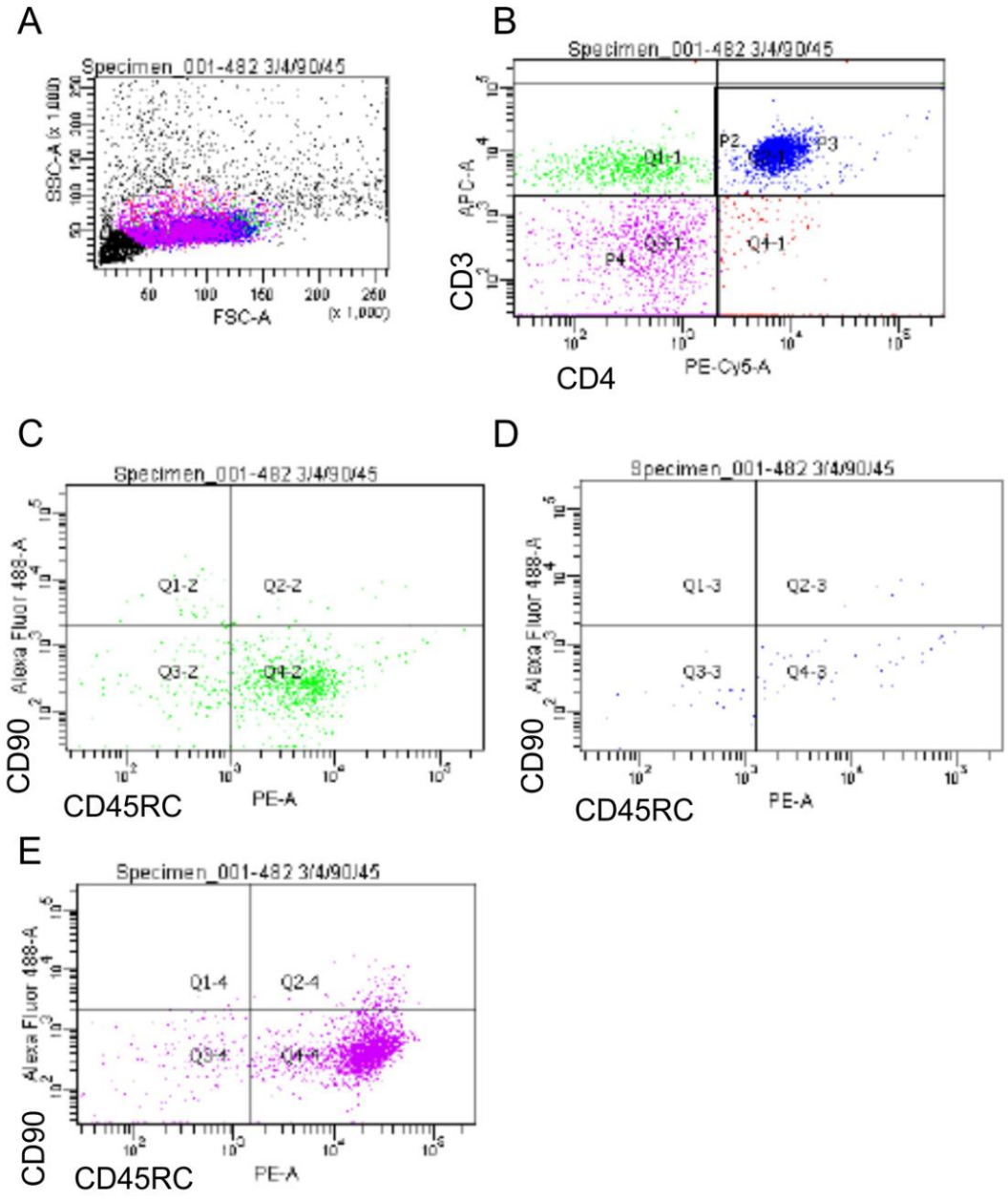

A
Specimen_001-482 3/4/8/25
SSC-A (x 1,000)
FSC-A (x 1,000)
B
Specimen_001-482 3/4/8/25
APC-A
CD3
Q1
P2
Q2
Q3
P5
Q4
CD4 PE-Cy5-A
C
Specimen_001-482 3/4/8/25
PE-Cy5-A
CD4
P3
Q2-1
P4
Q3-1
Q4-1
CD8a Alexa Fluor 488-A
D
Specimen_001-482 3/4/8/25
PE-Cy5-A
CD4
Q1-2
Q2-2
Q3-2
Q4-2
CD25 PE-A
E
Specimen_001-482 3/4/8/25
Alexa Fluor 488-A
CD8a
Q1-3
Q2-3
Q3-3
Q4-3
CD25 PE-A
F
Specimen_001-482 3/4/8/25
PE-Cy5-A
CD4
Q1-4
Q2-4
Q3-4
Q4-4
CD8a Alexa Fluor 488-A

Appendix C. Representative ZDF rat combination 1 flow cytometry plots. Definition of cell sample by light scatter; definition of viable cell population (A), definition of total T-lymphocytes ($CD3^+$) and non-T-lymphocytes ($CD3^-$) after gating on viable cell population (B), definition of helper ($CD3^+$ $CD4^+$) and cytotoxic ($CD3^+$ $CD8a^+$)T-lymphocytes after gating on T-lymphocyte ($CD3^+$) population (C), definition of activated ($CD3^+$ $CD4^+$ $CD25^+$) and unactivated ($CD3^+$ $CD4^+$ $CD25^-$) helper T-lymphocytes after gating on helper T-lymphocytes ($CD3^+$ $CD4^+$) (D), definition of activated ($CD3^+$ $CD8a^+$ $CD25^+$) and unactivated ($CD3^+$ $CD8a^+$ $CD25^-$) cytotoxic T-lymphocytes after gating on cytotoxic T-lymphocytes ($CD3^+$ $CD8a^+$) (E), and definition of monocytes/macrophages ($CD3^-$ $CD4^+$) and natural killer cells ($CD3^-$ $CD8a^+$) after gating on non-T-lymphocytes ($CD3^-$) (F).

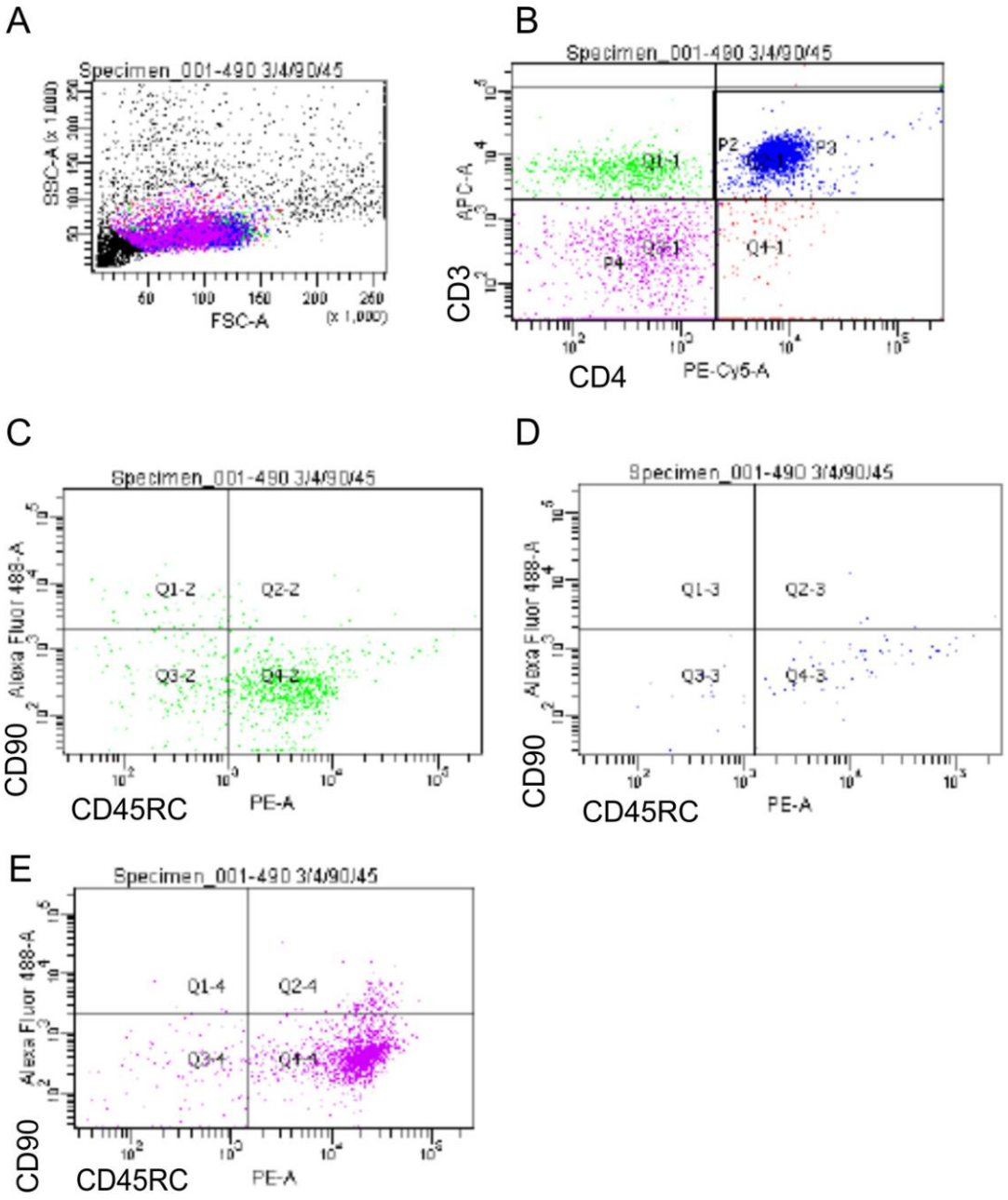

A
Specimen_001-491 3/4/8/25
SSC-A
FSC-A
B
Specimen_001-491 3/4/8/25
APC-A
CD3
CD4
PE-Cy5-A
Q1
Q2
Q3
Q4
P5
C
Specimen_001-491 3/4/8/25
PE-Cy5-A
CD4
CD8a
Alexa Fluor 488-A
Q1-1
Q2-1
Q3-1
Q4-1
P3
P4
D
Specimen_001-491 3/4/8/25
PE-Cy5-A
CD4
CD25
PE-A
Q1-2
Q2-2
Q3-2
Q4-2
E
Specimen_001-491 3/4/8/25
Alexa Fluor 488-A
CD8a
CD25
PE-A
Q1-3
Q2-3
Q3-3
Q4-3
F
Specimen_001-491 3/4/8/25
PE-Cy5-A
CD4
CD8a
Alexa Fluor 488-A
Q1-4
Q2-4
Q3-4
Q4-4

Appendix D. Representative lean ZDF rat combination 1 flow cytometry plots.

Definition of cell sample by light scatter; definition of viable cell population (A), definition of total T-lymphocytes ($CD3^+$) and non-T-lymphocytes ($CD3^-$) after gating on viable cell population (B), definition of helper ($CD3^+$ $CD4^+$) and cytotoxic ($CD3^+$ $CD8a^+$)T-lymphocytes after gating on T-lymphocyte ($CD3^+$) population (C), definition of activated ($CD3^+$ $CD4^+$ $CD25^+$) and unactivated ($CD3^+$ $CD4^+$ $CD25^-$) helper T-lymphocytes after gating on helper T-lymphocytes ($CD3^+$ $CD4^+$) (D), definition of activated ($CD3^+$ $CD8a^+$ $CD25^+$) and unactivated ($CD3^+$ $CD8a^+$ $CD25^-$) cytotoxic T-lymphocytes after gating on cytotoxic T-lymphocytes ($CD3^+$ $CD8a^+$) (E), and definition of monocytes/macrophages ($CD3^-$ $CD4^+$) and natural killer cells ($CD3^-$ $CD8a^+$) after gating on non-T-lymphocytes ($CD3^-$) (F).


A
Specimen_001-482 3/4/90/45
SSC-A (x 1,000)
FSC-A (x 1,000)
B
Specimen_001-482 3/4/90/45
APC-A
CD3
CD4
PE-Cy5-A
P2
P3
P4
Q1-1
Q3-1
Q4-1
C
Specimen_001-482 3/4/90/45
Alexa Fluor 488-A
CD90
CD45RC
PE-A
Q1-2
Q2-2
Q3-2
Q4-2
D
Specimen_001-482 3/4/90/45
Alexa Fluor 488-A
CD90
CD45RC
PE-A
Q1-3
Q2-3
Q3-3
Q4-3
E
Specimen_001-482 3/4/90/45
Alexa Fluor 488-A
CD90
CD45RC
PE-A
Q1-4
Q2-4
Q3-4
Q4-4

Appendix E. Representative ZDF rat combination 2 flow cytometry plots. Definition of cell sample by light scatter; definition of viable cell population (A), definition of total T-lymphocytes ($CD3^+$) and non-T-lymphocytes ($CD3^-$) after gating on viable cell population (B), definition of recent ($CD3^+$ $CD90^+$) and late (mature) ($CD3^+$ $CD90^-$) thymic emigrants after gating on T-lymphocyte ($CD3^+$) population (C), definition of naïve ($CD3^+$ $CD45RC^+$ $CD4^+$) and memory ($CD3^+$ $CD45RC^-$ $CD4^+$) helper T-lymphocytes after gating on helper T-lymphocytes ($CD3^+$ $CD4^+$) (D), definition of immature ($CD45RC^+$ $CD90^+$), mature ($CD45RC^+$ $CD90^-$), and total B-lymphocytes ($CD45RC^+$) after gating on non-T-lymphocytes ($CD3^-$) (E).


A
Specimen_001-490 3/4/90/45
SSC-A (x 1,000)
FSC-A (x 1,000)
B
Specimen_001-490 3/4/90/45
APC-A
CD3
CD4
PE-Cy5-A
P2
P3
P4
Q1-1
Q2-1
Q4-1
C
Specimen_001-490 3/4/90/45
Alexa Fluor 488-A
CD90
CD45RC
PE-A
Q1-2
Q2-2
Q3-2
Q4-2
D
Specimen_001-490 3/4/90/45
Alexa Fluor 488-A
CD90
CD45RC
PE-A
Q1-3
Q2-3
Q3-3
Q4-3
E
Specimen_001-490 3/4/90/45
Alexa Fluor 488-A
CD90
CD45RC
PE-A
Q1-4
Q2-4
Q3-4
Q4-4

Appendix F. Representative lean ZDF rat combination 2 flow cytometry plot.

Definition of cell sample by light scatter; definition of viable cell population (A), definition of total T-lymphocytes ($CD3^+$) and non-T-lymphocytes ($CD3^-$) after gating on viable cell population (B), definition of recent ($CD3^+$ $CD90^+$) and late (mature) ($CD3^+$ $CD90^-$) thymic emigrants after gating on T-lymphocyte ($CD3^+$) population (C), definition of naïve ($CD3^+$ $CD45RC^+$ $CD4^+$) and memory ($CD3^+$ $CD45RC^-$ $CD4^+$) helper T-lymphocytes after gating on helper T-lymphocytes ($CD3^+$ $CD4^+$) (D), definition of immature ($CD45RC^+$ $CD90^+$), mature ($CD45RC^+$ $CD90^-$), and total B-lymphocytes ($CD45RC^+$) after gating on non-T-lymphocytes ($CD3^-$) (E).