Effects of Specific Conjugated Linoleic Acid (CLA) Isomers on Insulin Resistance, and Skeletal Muscle AMP-Activated Protein Kinase-Alpha (AMPK-α) in *fa/fa* and Lean Zucker Rats

By

Robert Peter Diakiw

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

Department of Human Nutritional Sciences

University of Manitoba

Winnipeg, MB, Canada

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ABSTRACT

Effects of Specific Conjugated Linoleic Acid (CLA) Isomers on Insulin Resistance, and Skeletal Muscle AMP-Activated Protein Kinase-Alpha (AMPK-α) in *fa/fa* and Lean Zucker Rats

Robert P. Diakiw, M.Sc. Thesis,

Department of Human Nutritional Sciences

Conjugated linoleic acid (CLA) has been observed to reduce insulin resistance by improving glucose tolerance and reducing serum triglycerides and serum free fatty acids in certain animal models. Similarly, AMP-activated protein-kinase (AMPK) has been observed to mediate the action of antidiabetic drugs such as metformin and rosiglitazone by improving insulin resistance in animal models of Type 2 diabetes and obesity. Recently, studies have shown that CLA supplementation enhances adiponectin levels in adipose tissue and plasma, and in turn, plasma adiponectin has been observed to activate AMPK-α within skeletal muscle. Studies have also demonstrated that tumor necrosis factor-α (TNF-α) directly interferes with the insulin-signaling cascade at an early step and thus impairs insulin-stimulated glucose transport. High levels of TNF-α protein are associated with obesity, making TNF-α a possible target for CLA action.

As a result, we hypothesized that CLA would improve insulin sensitivity in the *fa/fa* Zucker rat by increasing skeletal muscle AMPK-α protein levels and activation, and concomitantly reducing TNF-α protein levels in skeletal muscle. Thus, the main objective of this study was to investigate the effects of an 8 week dietary supplementation with *cis*9,*trans*11 (c9,t11) and *trans*10,*cis*12 (t10,c12) CLA isomers, individually and in combination, on glycemic control, hyperlipidemia, skeletal muscle AMPK-α protein levels and activation, and TNF-α protein levels in *fa/fa* and lean Zucker rats.

The t10,c12 CLA isomer individually and in combination with other CLA isomers (purified 50:50 mixture with c9,t11 CLA and/or a two or four isomer CLA mixture) improved oral glucose tolerance and lowered estimated insulin resistance in the *fa/fa* Zucker rats. However, dietary CLA had no effect on fasting serum triglycerides or free fatty acids. Similarly, dietary CLA supplementation had no effect on skeletal muscle AMPK-α protein levels and activation, or TNF-α protein levels.

In conclusion, CLA mixtures and the t10,c12 CLA isomer reduced insulin resistance by improving glucose tolerance in the *fa/fa* rat, while the c9,t11 CLA isomer had no effect. However, these improvements were not coupled to changes in skeletal muscle AMPK-α protein levels or activation, or a reduction in TNF-α protein. Therefore, another unidentified mechanism must mediate CLA's effects on improved glucose tolerance and insulin resistance.

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ABBREVIATIONS

%CV	Percent coefficient of variation
10,12	*trans*10, *cis*12 CLA isomer diet
9,11	*cis*9, *trans*11 CLA isomer diet
A_1AR	A_1 adenosine receptors
ACC	Acetyl-CoA carboxylase
ACO	Acyl-CoA oxidase
Acyl CS	Acyl-CoA synthetase
AICAR	5-aminoimidazole-4-carboxamide ribonucleoside
AMP	Adenosine monophosphate
AMPK-α	Adenosine monophosphate-activated protein kinase-α
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
AUCg	Area under the curve for glucose
BIO	Bioriginals diet
BMI	Body Mass Index
BSA	Bovine serum albumin
BWA1433	1,3-dipropyl-8-(p-acrylic) phenylxanthine
c9,t11	*cis*9, *trans*11 CLA isomer
CAD	Coronary artery disease
CHD	Coronary heart disease
CLA	Conjugated linoleic acid
CPT-1	Carnitine palmitoyltransferase-1

CTL	Control diet
DHBS	3,5-dichloro-2-hydroxy-benzenesulfonic acid
EDL	Extensor digitorum longus
FER	Feed efficiency ratio
FFA	Free fatty acids
GLUT1	Glucose transporter -1
GLUT4	Glucose transporter -4
HDL	High-density lipoprotein
HOMA-IR	Homeostasis model assessment index of insulin resistance
IGF-1	Insulin-like growth factor-1
IR	Insulin receptor
IRS	Insulin receptor substrate
kDa	Kilodalton
LDL	Low-density lipoprotein
NCK	Nucheck diet
NEFA	Nonesterified fatty acids
NSB	Non-specific binding
ob/ob	Obesity gene
OCT	Optimal cutting temperature compound
OGTT	Oral glucose tolerance testing
Phospho-AMPK-α	Phosphorylated- adenosine monophosphate-activated protein kinase-α

PI3K	Phosphatidylinositol 3-kinase
POD	Peroxidase
PVDF	Polyvinylidene fluoride
PW	Pair weighed group
RIA	Radioimmunoassay
SAS	Statistical analysis system
SDS	Sodium dodecyl sulphate
SEM	Standard error of the mean
SH2	Src homology 2
t10,c12	*trans*10, *cis*12 CLA isomer
TAG	Triacylglycerides
TBHB	2,4,6-tribromo-3-hydroxy-benzoic acid
TBST	Tris buffered saline with Tween 20
TG	Triglyceride
Thr^{172}	Threonine 172
TNF-α	Tumor necrosis factor-α
TOG	Together diet
VLDL	Very-low-density lipoprotein
VO_2	Volume of oxygen
wt/wt	Weight by weight
ZDF	Zucker diabetic fatty

1. LITERATURE REVIEW

Introduction: Obesity and Type 2 Diabetes Mellitus

Worldwide, obesity and Type 2 diabetes mellitus are strongly interrelated diseases that contribute significantly to cardiovascular disease. Obesity is defined when a person has a Body Mass Index (BMI) of greater than 30 kg/m^2 due to the excessive accumulation of adipose tissue (WHO, 1998). Type 2 diabetes accounts for 90% of all diabetes mellitus cases and occurs when target cells in the body cannot sufficiently respond to insulin and there is impaired beta-cell function. Insulin resistance may be due to either low numbers or low binding affinity of insulin receptors or due to intracellular post-receptor defects (reviewed in Le Roith & Zick, 2001).

Obesity is associated with low insulin sensitivity, and reduced insulin sensitivity is a strong predictor of weight gain as stated by Ravussin & Gautier (1999). Obesity and metabolic inabilities related to insulin function may possibly aggravate each other, since there is a strong correlation between high levels of abdominal fat and low insulin sensitivity (Groop, 2000). The greatest risk factor for Type 2 diabetes is obesity, which is implicated in the development of over 75% of all cases (Shils *et al.*, 1999).

Many populations throughout the world have increased prevalence of obesity and Type 2 diabetes. It has been estimated that by 2025 approximately

300 million people will be obese and the numbers of people with diabetes will double, also reaching about 300 million people worldwide (WHO, 1998). Nearly, 65-75% of Type 2 diabetes would not occur if BMIs of the population were maintained at less than 25 kg/m^2 (Seidell, 1998). By 2010, it is predicted that approximately 3 million people will have diabetes in Canada (CDA, 2001).

The incidence of obesity and Type 2 diabetes can be attributed in part to the fact that caloric intake continues to rise and lifestyles are more sedentary. Genetics also play an important role and can contribute up to two-thirds of BMI variability (Ravussin & Bogardus, 2000). Aboriginals experience a three to five times higher incidence of Type 2 diabetes than the general Canadian population, due to genetic susceptibility, poor dietary habits, and a higher prevalence of central obesity (CDA, 2001).

Individuals affected with obesity and/or Type 2 diabetes require lifestyle modification. Diet can play an important role in the prevention and treatment of obesity and Type 2 diabetes. The focus of the literature review is to investigate the current state of knowledge surrounding the role of conjugated linoleic acid (CLA) in regulating metabolism and ultimately the treatment and prevention of Type 2 diabetes. Skeletal muscle accounts for the majority of insulin-stimulated glucose utilization and is a major site of insulin resistance in obesity and Type 2 diabetes (Manco *et al.*, 2000). AMP-Activated Protein Kinase (AMPK) is a downstream component of a protein kinase cascade that acts as an intracellular

energy sensor maintaining the energy balance within the cell. Activation of AMPK within skeletal muscle induces many metabolic changes that may be beneficial to subjects with Type 2 diabetes. It has been shown that leptin activates AMPK in skeletal muscle, thereby increasing fatty acid oxidation (Steinberg *et al.*, 2003). Adiponectin has been observed to activate AMPK in muscle to stimulate glucose and fatty acid usage (Thomas *et al.*, 2002; Yamauchi *et al.*, 2002). In addition, AMPK mediates the action of antidiabetic drugs such as metformin and rosiglitazone (Musi *et al.*, 2002; Fryer *et al.*, 2002). These results lead to the thought that AMPK activators may play a role as antidiabetic agents. However, the underlying mechanisms for these effects remain largely undefined. Thus, skeletal muscle AMP-Activated Protein Kinase-α (AMPK-α) will be reviewed to determine its role in the treatment and prevention of Type 2 diabetes.

Insulin Resistance

The decreased ability of insulin to act on peripheral tissues is commonly termed "insulin resistance". Insulin resistance is defined as a decreased biological response to normal concentrations of circulating insulin needed in the transport of glucose from the bloodstream into peripheral tissues (Shulman, 2000). Insulin resistance can also be viewed as the dysfunction of pancreatic β-cells, to produce sufficient amounts of insulin to overcome insulin resistance in

the peripheral tissues resulting in a functional deficiency of insulin (Shulman, 2000).

The ability of insulin to stimulate glucose uptake varies widely from person to person, and these differences, as well as how the individual attempts to compensate for them, are of fundamental importance in the development and clinical course of diabetes. There is considerable evidence that Type 2 diabetes results from a failure on the part of pancreatic β-cells to compensate adequately for the defect in insulin action in insulin-resistant individuals (Shulman, 2000).

Individuals that are insulin resistant and hyperinsulinemic are more likely to be hypertensive and have a dyslipidemia characterized by high plasma triglyceride (TG), lower levels of high-density lipoprotein (HDL) cholesterol concentrations, and higher amounts of low-density lipoprotein (LDL) with smaller LDL particles. All of these changes have also been shown to increase the risk of coronary heart disease (CHD) and have been termed "Syndrome X" (Reaven, 1993). Cardiovascular disease accounts for 36 percent of all deaths in Canada and along with stroke is the number one cause for hospitalization and death in Canada (Heart and Stroke, 2003).

Insulin resistance seems to be necessary for the components of Syndrome X to develop. In order to understand the pathophysiology of Syndrome X, it must be realized that not all tissues respond similarly to insulin in the same

individual. For example, although the ability of insulin to stimulate glucose uptake by muscle and inhibit adipose tissue lipolysis is highly correlated within an individual, insulin resistance at the level of the muscle and adipocyte does not mean that insulin action on the kidney is impaired. For example, the compensatory hyperinsulinemia associated with muscle insulin resistance acts on a normally insulin-sensitive kidney to increase sodium re-absorption and decrease uric acid clearance. This contributes to the hypertension and increase in plasma uric acid concentration seen in patients with Syndrome X (Reaven, 1993).

Insulin resistance and compensatory hyperinsulinemia are associated with a variety of abnormalities, including varying degrees of glucose intolerance, higher plasma TG and lower HDL cholesterol concentrations, smaller, denser LDL particles, exaggerated postprandial lipemia, hyperuricemia, and high blood pressure. These abnormalities tend to cluster in the same individual, and represent major risk factors for the development of CHD.

In most non-hepatic tissues, insulin increases glucose uptake by increasing the number of plasma membrane glucose transporters (GLUTs). Increases in the plasma membrane content of transporters stem from an increase in the rate of recruitment of new transporters to the plasma membrane, deriving from a special pool of preformed transporters localized in the cytoplasm. GLUT1 is present in most tissues, while GLUT4 is found in heart, adipose tissue

and skeletal muscle (Kido *et al.*, 2001). Signaling through the insulin pathway is critical for the regulation of intracellular and blood glucose levels and the prevention of diabetes. Insulin binds to its receptor leading to the autophosphorylation of the β-subunits and the tyrosine phosphorylation of insulin receptor substrates (IRS). IRS phosphorylates the Src homology 2 (SH2) domain of Shp2, a tyrosine phosphatase, and the Src homology 3 (SH3) domain of the adaptor molecule growth factor receptor-bound protein 2 (Grb2). Activated Grb2 recruits the son of sevenless homolog 1 (Sos1) that, in turn, activates the Ras signaling pathway and gene transcription, which activates many signaling cascades. IRS also activates phosphatidylinositol 3-kinase (PI3K) through its SH2 domain, thus increasing the intracellular concentration of Phosphatidylinositol bisphosphate (PIP_2) and phosphatidylinositol phosphate (PIP). This rise in phosphorylated inositol levels, in turn, activates phosphatidylinositol phosphate-dependent kinase-1 (PDK-1), that subsequently activates Akt/PKB an important regulator of glucose homeostasis. Akt activity results in the translocation of the glucose transporter-4 (GLUT4) and to a lesser extent glucose transporter-1 (GLUT1) from cytoplasmic vesicles to the cell membrane (Kido *et al.*, 2001).

Thus, it is concluded that insulin resistance and its associated abnormalities are of utmost importance in the pathogenesis of Type 2 diabetes, hypertension, and coronary heart disease.

Insulin Resistance and Hyperlipidemia

Hyperlipidemia is a lipid profile of decreased HDL cholesterol levels (a significant risk factor for heart disease), and increased serum very-low-density lipoprotein (VLDL) cholesterol, serum TG concentrations and smaller low-density lipoprotein cholesterol particles (Kreisberg & Oberman, 2003). These are typical of a person with Type 2 diabetes.

The three major components of the hyperlipidemia of insulin resistance are the increased TG's, decreased HDL cholesterol, and changes in the composition of LDL cholesterol (Kreisberg & Oberman, 2003). Hyperinsulinemia and the central obesity that typically accompanies insulin resistance are thought to lead to overproduction of VLDL cholesterol. This results in more TG-rich particles, fewer HDL particles, and more, small, dense LDL (Kreisberg & Oberman, 2003). Postprandial TG levels and postprandial remnants also may contribute to increased coronary artery disease (CAD) risk in individuals with insulin resistance.

Deficiency of lipoprotein lipase, an insulin-sensitive enzyme, might explain the abnormal levels of remnant particles in insulin resistance (Kreisberg & Oberman, 2003). The potential benefits of successful treatment of hyperlipidemia are illustrated by clinical trials in patients with the hyperlipidemia characteristic of insulin resistance (i.e., normal or only moderately elevated LDL, elevated VLDL,

and low HDL) (Kreisberg & Oberman, 2003). Both weight loss and exercise can improve insulin resistance and associated dyslipidemia. In patients with Type 2 diabetes, certain antidiabetic therapies can also improve the lipid profile by improving insulin resistance.

Insulin Resistance and Tumor Necrosis Factor-alpha (TNF-α)

Tumor necrosis factor-alpha (TNF-α), known as cachectin, is a smaller cytokine that binds to the tumor necrosis factor receptors producing a vast array of effects (Greiwe *et al.*, 2001). TNF-α is produced by several different cell types, including lymphocytes, neutrophils and macrophages, and can modulate many immune and inflammatory functions, while having the ability to inhibit tumor growth.

Initially, this cytokine is expressed as a 26 kDa membrane protein that is subsequently cleaved by a metalloproteinase enzyme (TNF-α converting enzyme) to a 17 kDa or 19 kDa soluble monomer. The 17 kDa or 19 kDa soluble monomer is TNF-α in its physiologically active form (Pallandino *et al.*, 2003).

TNF-α deficient obese mice have lower plasma levels of free fatty acids (FFA), were protected from the obesity-related reduction in insulin receptor signaling in muscle and fat tissues, and had improved *in vivo* insulin sensitivity (Uysal *et al.*, 1997). These results indicate that TNF-α protein is an important

mediator of insulin resistance in rodent obesity through its effects on several important sites of insulin action. Because TNF-α is produced by adipose tissue and because serum levels of TNF-α are very low, it is reasonable to suppose that this cytokine acts directly via an autocrine (adipocytes) or paracrine (skeletal muscles in close proximity to the adipose tissue) loop rather than an endocrine effect. Another alternative may be that TNF-α acts directly on adipose tissue to stimulate the production of other molecules that indirectly acts on muscle (Hotamisligil *et al.*, 1996). FFA may be involved in such a process as their release by adipocytes is stimulated by TNF-α (and reduced in TNF-α-deficient obese mice) (Uysal *et al.*, 1997), and FFA are well known to inhibit insulin action on skeletal muscle. Finally, a last mechanism by which TNF-α may play an important role in insulin resistance is by its expression in muscle (Saghizadeh *et al.*, 1996). Indeed, it has been reported that TNF-α is expressed at a higher level in muscle tissue and in cultured muscle cells from insulin-resistant and diabetic subjects, and there is a significant inverse relationship between muscle TNF-α expression and *in vivo* insulin sensitivity (Saghizadeh *et al.*, 1996). If crosstalk between (visceral) adipose tissue and skeletal muscle is mediated by TNF-α via an endocrine mechanism, a negative correlation between circulating TNF-α levels and individual insulin sensitivity indices would be expected (Saghizadeh *et al.*, 1996).

Greiwe *et al.*, (2001) found that in a cross-sectional comparison of young and frail elderly subjects, TNF-α protein levels were higher in the elderly. After 3

months of resistance exercise, TNF-α protein levels decreased in conjunction with increases in strength and protein synthesis.

Not many studies have looked at animal models with regards to TNF-α protein levels in muscles. However, the cytokine TNF-α has been proposed as a critical mediator in insulin resistance induction (Hotamisligil *et al.*, 1994). The fact that TNF-α protein is high in muscle and adipose tissue in obesity and diabetes led to the hypothesis that this may be a key factor in insulin resistance development. Hotamisligil *et al.* (1994) support this possibility based on studies of genetically obese Zucker *fa/fa* rats, which showed systemic administration of monoclonal antibodies that neutralize TNF-α protein reversed insulin resistance. However, the administration of similar antibodies (antibody CDP571) to subjects with Type 2 diabetes did not result in any significant change in insulin sensitivity and glycemic control (Ofei *et al.*, 1996).

Studies have demonstrated that TNF-α directly interferes with the insulin-signaling cascade at an early step (Sethi & Hotamisligil, 1999). *In vitro* studies have shown that TNF-α has been connected to several mechanistic steps of importance for the development of the metabolic syndrome. Proposed mechanisms are impaired auto-phosphorylation of insulin receptor (IR) and its substrate IRS-1; externalization of the insulin-induced GLUT4; and inhibition of lipoprotein lipase (Sethi & Hotamisligil, 1999).

Sethi & Hotamisligil (1999) reviewed a number of *in vitro* studies of adipocytes which suggested that TNF-α may act directly to down-regulate GLUT4 gene expression, thereby decreasing insulin- stimulated glucose transport. Interestingly, GLUT4 content is reduced in adipocytes from obese Type 2 diabetic subjects. Whether this is the main mechanism of TNF-α action in human adipose tissue in obesity remains unclear. However, in obese rodents lacking TNF-α, adipose levels of GLUT4 remain similar to their normal counter-parts. This suggests that at least in adipose tissue *in vivo*, TNF-α acts via a mechanism independent of GLUT4 expression. TNF-α has also been shown to act on the proximal steps of insulin signaling. In cultured adipocytes, TNF-α increases the phosphorylation of IRS-1 at serine residues. This converts this multi-functional docking protein into an inhibitor of the IR tyrosine kinase. Such a modification of IRS-1 has been observed in obesity insulin resistance and is sufficient to block the downstream events of IR signaling, including the association of IRS-1 with PI3K. TNF-α playing a role in these actions of insulin resistance *in vivo* is supported by studies in which both pharmacological and genetic blockade of TNF-α function results in increased signaling capacity of insulin receptors. These findings support the hypothesis that TNF-α is a mediator of obesity-linked insulin resistance.

Conversely, investigators have found that in some cases TNF-α can stimulate glucose uptake in skeletal muscles. Both human skeletal muscle cells and rat myoblasts have been used to determine whether TNF-α causes

resistance to glucose uptake stimulated by insulin signaling pathways in skeletal muscle. Studies with rat L6 myoblasts, which contain receptors for TNF-α have shown that TNF-α weakly stimulated glucose uptake by 3-fold after 48 hours of incubation (Yamasaki *et al.*, 1996).

Ciaraldi *et al.* (1998) looked at the effects of TNF-α on glucose uptake and glycogen synthase activity with both acute and prolonged exposures in human skeletal muscle cell cultures from both non-diabetic subjects and subjects with Type 2 diabetes. In muscle cells from non-diabetic subjects, an acute 90-min exposure to TNF-α stimulated glucose uptake to a greater extent than did insulin. The acute glucose uptake response to TNF-α in cells from subjects with Type 2 diabetes was also greater than with insulin stimulation. When these same cells from non-diabetic subjects were continually exposed to TNF-α for a duration of 24 hours, it resulted in a further stimulation of glucose uptake. However, in cells from the subjects with Type 2 diabetes, continued TNF-α exposure for a duration of 24 hours did not significantly further glucose uptake. Glucose transporters were also examined. After chronic TNF-α exposure, the level of GLUT1 protein was elevated 4.6-fold in cells from non-diabetic subjects compared to 1.7-fold in cells from subjects with Type 2 diabetes, while no effects in either cells from non-diabetic subjects or Type 2 diabetic subjects were seen in the levels of GLUT4 protein. In contrast, acute TNF-α treatment had no effect on glycogen synthase in cells from either non-diabetic subjects or those with Type 2 diabetes, whereas prolonged TNF-α exposure reduced glycogen synthase from both groups. Thus,

both acute and prolonged treatment with TNF-α showed an up-regulation of glucose uptake activity in cultured human muscle cells, but reduced glycogen synthase activity.

Therefore it seems that TNF-α may interfere with insulin signaling, impairing insulin-stimulated glucose transport. This suggests that TNF-α may be a mediator of obesity-linked insulin resistance.

Adenosine Monophosphate-Activated Protein Kinase (AMPK)

AMPK is similar to the sucrose non-fermenting 1 protein kinase in yeast. It plays a major role in the adaptation to nutrient stress (Hardie *et al.*, 1998). AMPK is a trimeric enzyme consisting of an α subunit, which contains the catalytic domain, and beta (β) and gamma (γ) subunits, (Kemp *et al.*, 1999).

AMPK is activated through phosphorylation by one or more upstream AMPK kinases. In the absence of phosphorylation, there is no detectable activity of AMPK towards any substrates. Phosphorylation of AMPK occurs in the α subunit at threonine 172 (Thr^{172}), which lies in the activation loop. However, AMPK is also regulated by AMP through a direct allosteric activation mechanism that senses increases in the AMP:ATP ratio as well as creatine:phosphocreatine (Kemp *et al.*, 1999).

There are two known isoforms of the α subunit, α1 and α2. These isoforms transfer a high-energy phosphate from ATP to serine and threonine on a number of different proteins. Residue Thr^{172} is found within the α subunit, which functions as a phosphorylation activation site for one or more upstream AMPK kinase such as Ca^{2+}/calmodulin-dependent protein kinase kinase beta (CaMKKβ). Skeletal muscle contains both the α1 and α2 catalytic isoforms. Both isoforms have a molecular mass of approximately 63 kDa (Thornton *et al.*, 1998).

Both α1 and α2 isoforms are distributed throughout the cytosol of skeletal muscle. There is also evidence that a substantial percentage of α2 AMPK is associated with nuclei (Ai *et al.*, 2002). In muscles composed of both slow-twitch (type I) and fast-twitch (type IIa and IIb) fibers, the α1 and α2 isoforms are both present.

AMPK Activity Regulators in Skeletal Muscle

AMPK is linked to energy, or more so, energy depletion. The catalytic activity of AMPK increases when the cell is under conditions associated with energy depletion. This activation of kinase activity is believed to primarily switch on pathways for ATP regeneration. This is why AMPK activity in contracting skeletal muscles is increased, due to the decreases in muscle energy stores. In addition to contracting skeletal muscles, hypoxia, hyperosmolarity and

uncouplers of oxidative phosphorylation increase activity of AMPK in skeletal muscle (Hardie & Hawley, 2001). Recently it has also been shown that the tumor suppressor LKB1 a serine/threonine kinase may regulate AMPK activity in skeletal muscle of LKB1 knockout mice (Sakamoto *et al.*, 2005).

Nielsen *et al.* (2003), states that in human skeletal muscle, exercise activates AMPK in an isoform specific, intensity and time dependent manner. At lower work intensities such as 50% VO_2 peak, no increase in AMPK activity associated with the α1 or α2 subunit is detectable. Conversely, if the exercise is sustained for approximately 3.5 hours, AMPK α2 activity is increased. At higher intensities of approximately 65–90% VO_2 peak, an increase in AMPK α2 activity above basal measurement is detected as soon as 5 minutes into the exercise bout, where as α1 activity is only increased when maximal sprint exercise is performed.

However, there are many factors that can modify the extent of AMPK activation in response to various stimuli. These include high muscle glycogen, glycogen super-compensation, muscle fibre type and being in a fed or fasted state. Subjects who have high muscle glycogen content, showing the lack of functional glycogen phosphorylase, have inflated muscle AMPK-α_2 activation and glucose disposal during exercise (Nielsen *et al.*, 2002). However, glycogen super-compensation in rat skeletal muscle blunts the acute increases in 5-aminoimidazole-4-carboxamide ribonucleoside (AICAR), which is a compound that is taken up into skeletal muscle and metabolized by adenosine kinase to

form 5-amino-4-imidazolecarboxamide riboside 5′-monophosphate, the monophosphorylated derivative that mimics the effects of AMP on AMPK (Hayashi *et al.*, 1998), and contraction-stimulated AMPK activity and glucose uptake (Wojtaszewski *et al.*, 2002).

AMPK is affected by the nutritional state as well. In rat epitrochlearis and flexor digitorum brevis muscles, stimulation of AMPK activity by muscle contraction is higher in the fasted state compared with the fed state. In contrast, AICAR increases AMPK activity in the rat epitrochlearis and flexor digitorum brevis muscles to a similar extent regardless of the nutritional state (Ai *et al.*, 2002). In epitrochlearis muscles, AICAR and contraction simulated glucose uptake is higher in the fasted state as well, but in flexor digitorum brevis and soleus muscle, the increase in glucose uptake in response to contraction is not affected by nutritional state.

Activation of AMPK by different stimuli also varies depending on muscle fibre type (Ai *et al.*, 2002). In rat muscles, contraction produces a strong activation of AMPK in epitrochlearis (type IIb fibres), flexor digitorum brevis (type IIa fibres) and soleus (type I fibres), while AICAR increases AMPK activity in both epitrochlearis and flexor digitorum brevis but has no effect in soleus. Similarly, AICAR stimulates glucose uptake in epitrochlearis and flexor digitorum brevis muscles but has no effect in soleus muscle (Ai *et al.*, 2002).

AMPK and Skeletal Muscle Fatty Acid Oxidation

In skeletal muscle, acetyl-coA carboxylase (ACC) is the rate-limiting enzyme in fatty acid synthesis and its activity is regulated by phosphorylation. Phosphorylation of ACC occurs through the actions of AMPK. When AMPK is phosphorylated or in its activated form due to the actions of protein hormones such as leptin and adiponectin (Ruderman *et al.*, 2003), it phosphorylates ACC and thus, reduces fatty acid synthesis.

ACC is the enzyme responsible for converting acetyl-CoA to malonyl-CoA and malonyl Co-A is the first intermediate in the formation of long-chain fatty acids (Hardie *et al.*, 1997). Phosphorylation and therefore inactivation of ACC lead to a decrease in the concentration of malonyl-CoA, a potent inhibitor of carnitine palmitoyltransferase 1 (CPT-1) (Ruderman *et al.*, 2003). With a drop in the inhibition of CPT-1, there is a concomitant increase in β-oxidation of fatty acids within the mitochondria. An increase in fatty acid oxidation, like increases in glycolysis, will lead to increases in ATP production. Thus, inhibition of long-chain fatty acid entry into the mitochondria is relieved and fatty acid oxidation is increased through the activation of AMPK.

AMPK and Skeletal Muscle Glucose Uptake

AMPK has been linked with muscle glucose uptake. Evidence in support of a role for AMPK in contraction-stimulated glucose transport comes from

studies using AICAR. AICAR infusion enhances insulin-stimulated glucose transport in perfused rat hindlimb skeletal muscles (Merrill *et al.*, 1997). Insulin, muscle contraction and AICAR all have been shown to increase glucose transport in isolated rat epitrochlearis muscles incubated *in vitro*. The increase in glucose transport with the combination of maximal AICAR plus maximal insulin treatments is partially additive, while there is no additive effect on glucose transport with the combination of AICAR plus contraction (Hayashi *et al.*, 1998).

As in contraction, treatment of rodent and human skeletal muscle with AICAR elicits the translocation of GLUT4 to the plasma membrane in cardiac and skeletal muscle *in vitro* (Kurth-Kraczek *et al.*, 1999). In certain skeletal muscle cells, overexpression of active AMPK stimulates glucose uptake accompanied by GLUT1 and GLUT4 translocation. This shows that AMPK is sufficient to stimulate glucose uptake and that AMPK mediated glucose uptake involves glucose transporter translocation (Fryer *et al.*, 2002).

However, AICAR does not have strict specificity to AMPK (Vincent *et al.*, 1991; Young *et al.*, 1996). Musi *et al.* (2001) has shown that iodotubercidin and adenosine-9-d-arabino-furanoside, which are alleged AMPK inhibitors (not specific to AMPK), inhibit AICAR-induced activation of AMPK, however, they do not inhibit contraction-induced AMPK activation in skeletal muscle. This suggests that there may be other mechanisms in addition to AICAR stimulated AMPK activation that regulates contractile glucose transport.

AMPK Targeted for the Treatment of Type 2 Diabetes

AMPK activation produces many metabolic changes that may be beneficial to individuals with Type 2 diabetes. There is evidence that chronic activation of AMPK via AICAR treatment improves lipid homeostasis and glucose, and insulin resistance in animal models of diabetes and obesity (Iglesias *et al.*, 2002; Olsen *et al.*, 2002). When activated, AMPK phosphorylates and inhibits ACC and phosphorylates and activates malonyl-CoA decarboxylase, leading to a decrease in the concentration of malonyl-CoA and an increase in fatty acid oxidation (Ruderman *et al.*, 2003). AMPK also decreases fatty acid incorporation into glycerolipids (Park *et al.*, 2002).

It has also been shown that leptin activates AMPK in skeletal muscle, thereby increasing fatty acid oxidation (Steinberg *et al.*, 2003), whereas adiponectin activates AMPK in muscle to stimulate glucose and fatty acid usage (Thomas *et al.*, 2002; Yamauchi *et al.*, 2002). In addition, AMPK mediates the action of antidiabetic drugs such as metformin and rosiglitazone (Musi *et al.*, 2002; Fryer *et al.*, 2002). This leads to the proposal that AMPK activators may play a role as antidiabetic agents. However, the underlying mechanisms for these effects remain largely undefined.

Conjugated Linoleic Acid (CLA)

CLA refers to a group of polyunsaturated fatty acids that exist as positional and stereoisomers of conjugated dienoic octadecadienoate (18:2). The main isomer in foods is *cis*9,*trans*11 (c9,t11) CLA (Ma *et al.*, 1999), followed by t7,c9 CLA, c11,t13 CLA, c8,t10 CLA, and the t10,c12 CLA isomers (Fritsche *et al.*, 1999). The three dimensional stereo-isomeric configuration of CLA may be in combinations of *cis* and/or *trans* configurations.

The major foods that provide CLA are milk and dairy products (containing about 0.9%), ruminant and other meat products (containing about 0.4%), and cakes and pastries containing dairy products (with about 0.3%) (Gunstone,2000). For any individual, CLA intake will depend chiefly on the consumption of these foods and will obviously be lower for those who avoid meats and/or dairy products. In Germany, average intakes of CLA were found to be 430 and 350 mg/day for men and women, respectively, while in the USA, figures of 52-137 mg/day were calculated for men and women together, and in Finland levels of 40-310 mg/day have been reported (Gunstone, 2000). Gunstone (2000) suggests that it may not be possible to raise daily intake above 500 mg by normal dietary methods, since our consumption of dairy products and red meat has declined due to vegetarianism and health concerns. Indeed the intake of CLA is lower than it was a generation ago.

By adjusting animal diets, it may be possible to raise CLA levels in milk and in meat. CLA levels in milk fats vary with diet, stage of lactation, breed, and production management, and it may be possible raise average values to nearer the upper figures. Gunstone (2000) mentions that when CLA from an alkali-isomerised oil was incorporated into the diet of fast-growing chicks (broilers), the body lipids contained ~10% of CLA. Similar results have been reported with hen eggs and these materials could be used as a convenient way of increasing CLA in the human diet. Beyond these, it would be necessary to take the commercial CLA material directly. This is readily available in encapsulated form.

The CLA isomers present in eleven cheeses that had a 0.38-0.74% CLA content was mainly c9,t11 CLA (78-84%) acid accompanied by t7,c9 CLA, t8,c10 CLA, t11,c13 CLA, c12,t14 CLA dienes and all their *trans,trans* isomers (Gunstone, 2000). As already reported, the t7,c9 CLA is present in cows milk, cheese, beef, human milk and human adipose tissue at levels between 3 and 16% of total CLA. CLA present in human tissue contains all four c9,t11 diene isomers with the *cis,trans* and *trans,trans* isomers predominating and the *trans,cis* and *cis,cis* isomers as minor components (Chin *et al.*, 1992). CLA is present in human milk fat (2-6 mg/g) but absent from infant formula (Gunstone, 2000). The CLA in human milk fat is partly of dietary origin (~30%) but is also mobilized from body stores (Gunstone, 2000).

Rumenic acid (9,11-18:2) is produced by ruminants through enzymatic isomerisation of linoleic acid in the rumen followed by hydrogenation leading first to vaccenic (*trans*11-18:1) and finally to stearic acid (18:0) (Gunstone, 2000). In both ruminants and non-ruminants there is also an alternative pathway to CLA resulting from the action of the ubiquitous delta 9 desaturase on t11-18:1 and other *trans* monoenes. These *trans* monoene acids result from rumen biohydrogenation of linoleic acid or they are ingested in the diet as partially hydrogenated vegetable oils.

Rumenic acid is the only major conjugated 18:2 acid in ruminant and other animal fats. It is accompanied by several other CLA isomers at lower concentrations. The term 'conjugated linoleic acid' is now used to describe the mixed conjugated dienes obtained from both biological and chemical sources.

CLA isomers are also being produced for human ingestion by alkali-isomerisation of either linoleic acid or sunflower or safflower oils, which are rich in linoleic acid, yet contain virtually no linolenic acid, and are low in phospholipids and sterols (Gunstone, 2000). The products first obtained were complex mixtures containing mainly *cis,trans* isomers with lower levels of *cis,cis* and *trans,trans* compounds. Also, the major components (c9,t11 CLA and t10,c12 CLA dienes) were accompanied by other isomers resulting from further isomerisation of the major products. Now, improved procedures have been developed and patented. The reaction is effected with KOH (or other base) in

propylene glycol at ~150°C, ambient pressure, and inert gas for 2-6.5 hours, and under these conditions isomerisation is 90-99.5% complete. Recovery of the final product involves molecular distillation (190°C for one minute) and deodorization at 120-170°C (preferably ~150°C) for 2 hours. Under these conditions, it is claimed that the product is "pure", containing at least 50% of the 9,11 and 10,12 acids with less than 1% of other isomers (along with palmitic, stearic, and oleic acids) (Gunstone, 2000). Propylene glycol is preferred as solvent over ethylene glycol because of its lower toxicity and because reaction in this solvent proceeds more quickly at a lower temperature and furnishes a product with less color.

There has been lots of interest in obtaining individual isomers, which is now possible, if only for research purposes. It is also possible to concentrate the c9,t11 CLA or t10,c12 CLA acid from the commercial mixture described in the previous paragraph by extensive crystallization or by using enzymes which can distinguish between the CLA isomers (Gunstone, 2000).

CLA and Hyperlipidemia

Free fatty acids (or non-esterified fatty acids), triglycerides and cholesterol are all parameters of hyperlipidemia. Studies have shown that CLA mixture-fed Zucker diabetic fatty (ZDF) rats had reduced plasma free fatty acid concentrations (Houseknecht *et al.*, 1998), as well as reduced serum triglyceride

and non-esterfied fatty acid concentrations (Ryder *et al.*, 2001). The obesity (*ob/ob*) gene mice fed the c9,t11 CLA isomer they had reduced serum triglycerides and non-esterfied fatty acids, while those fed the t10,c12 CLA isomer, did not (Tsuboyama-Kasaoka *et al.*, 2000).

There is a growing body of evidence that CLA reduces atherosclerotic plaque formation in experimental animals (Houseknecht *et al.*, 1998). One study showed that when CLA mixture (0.5 g/rabbit/day) was added to a hypercholesterolemic diet and fed to rabbits for 22 weeks, serum triglycerides and low-density lipoprotein cholesterol levels were significantly reduced compared with rabbits fed a diet without CLA (Lee *et al.*, 1994). Nicolosi *et al.* (1997) fed hamsters (10/group) a diet containing 10% coconut oil and 0.25-5.0% CLA mixture, or 5% linoleic acid in the control diet. The control diet resulted in plasma cholesterol and triglyceride levels of 17.8 ± 0.62 and 12.4 ± 2.39 mm/L respectively. The area of aorta covered with lipid was 53 ± 14 ($\mu m^2/mm^2$ x 100). On the diet with the lowest CLA concentration (0.25%), plasma cholesterol and triglyceride levels were reduced by 26% and 58%, respectively, and aortic fatty streak area was reduced by 19%. A subsequent study (Wilson *et al.*, 1999) found a 47% reduction in aortic fatty streak area of hamsters fed 1% CLA mixture. Plasma LDL oxidation was also reduced significantly.

In contrast, Munday *et al.* (1999) studied three groups of twenty C57BL/6 mice fed atherogenic diets containing 5 g CLA mixture/kg, 2.5 g CLA + 2.5 g

linoleic acid/kg or 5 g linoleic acid/kg. All diets were fed for 15 weeks and contained 145 g/kg triacylglycerol, 5 g/kg free fatty acids, 10 g/kg cholesterol and 5 g/kg cholic acid. At the completion of the experimental period, when data from both groups fed on CLA were combined, dietary CLA mixture did not produce significant differences in body weight, serum total cholesterol concentration or serum HDL-cholesterol concentration. However, mice receiving CLA mixture developed a significantly higher serum HDL-cholesterol: total cholesterol ratio and a significantly lower serum triacylglycerol concentration than controls. In contrast to the protective effects of CLA on atherosclerotic plaque formation in rabbits and hamsters, CLA induced the formation of aortic fatty streaks in C57Bl/6 mice fed an atherogenic diet.

In summary, CLA appears to exert differential effects on lipid profiles as well as atherogenic markers in various animal models.

CLA and Insulin Resistance

Insulin resistance is related to glycemic control (glucose tolerance) in that low glucose tolerance reflects a state of poor insulin response by target cells. It has been reported that a mixture of dietary CLA isomers normalized impaired glucose tolerance and delayed the development of hyperglycemia in obese ZDF rats, in the same manner as troglitazone, an insulin sensitizer (Houseknecht *et al.*, 1998). ZDF rats spontaneously develop diabetes at 7–12 weeks due to beta-

cell decompensation, and Houseknecht *et al.* (1998) compared a corn oil (5%) plus lard (1.5%) control diet to a CLA (1.5%) diet and a troglitazone (0.2%) treatment. Troglitazone is a member of the thiazolidinedione class of drugs, which act as insulin sensitizers. These diets were fed for two weeks, commencing at 6 weeks of age. Rats fed the CLA mixture or thiazolidinedione diet exhibited significantly reduced fasting glucose and insulinemia.

In another study by Ryder *et al.* (2001), 7 week old ZDF and lean Zucker rats were fed a diet containing a 1.5% mixture of c9,t11 CLA and t10,c12 CLA (50:50) or 1.5% of the c9,t11 CLA isomer alone for 2 weeks. It was observed that along with improved glucose tolerance, insulin-stimulated glucose transport was improved as well in soleus muscle, and insulin-stimulated glycogen synthase activity was improved in soleus and extensor digitorum longus (EDL) muscles of 50:50 rats (47.0% c9,t11 CLA and 47.9% t10,c12 CLA isomers). The balance of CLA isomers in the 50:50 supplement include: 2.6% c9,c11 + c10,c12; 1.6% t9,t11 + t10,t12; and 0.8% other CLA isomers. However, these effects were not observed in rats fed the c9,t11 CLA diet alone, suggesting that the effects are specific to the t10,c12 CLA isomer.

Roche *et al.* (2002) studied male *ob/ob* mice fed either a 0.45% c9,t11 CLA isomer or a 0.45% t10,c12 CLA isomer. They found that the c9,t11 CLA diet significantly reduced serum TAG and FFA serum concentrations, while having no significant effect on serum glucose or insulin concentrations. In contrast, mice fed

the t10,c12 diet became severely insulin resistant, with marked increases in serum glucose and insulin concentrations. It was found that serum TAG and FFA levels were not further increased in the t10,c12 diet fed *ob/ob* mice despite their marked insulin resistance. This study showed that the t10,c12 CLA isomer was responsible for insulin resistance at least in the *ob/ob* mouse.

In another study, C57BL/6J mice were fed linoleic acid isomerized to a CLA mixture (34% c9,t11, t9,c11; 36% t10,c12; 3% c9,c11, c10,c12; 2% t9,t11, t10,t12 from total fatty acids). CLA mixture fed mice showed increases of fasting and feeding insulin concentrations by 4-fold and 8-fold, respectively. Both fasting and feeding plasma leptin levels also decreased in CLA mixture fed mice by 49% and 79%, respectively. As well, CLA mixture supplementation showed a marked downregulation of GLUT4 mRNA by 91% in parametrial white adipose tissue. These results suggested that insulin resistance might develop in CLA mixture fed mice (Tsuboyama-Kasaoka. 2000).

Brown *et al.* (2003) found that physiological levels (e.g., 10–30 μM) of t10,c12 CLA can act as an antiadipogenic nutrient in differentiating human preadipocytes. However, this effect is highly isomer specific, and previous work by Brown *et al.* (2001) demonstrated that the level used is critical in eliciting a TG-lowering effect. Brown *et al.* (2003) suggested it is likely that the t10,c12 isomer of CLA that reduces TG accumulation in differentiating preadipocytes by reducing fatty acid uptake and altering fatty acid metabolism in a way that favors

less oxidation, desaturation, and esterification of long chain fatty acids. Furthermore, the t10,c12 isomer of CLA dramatically inhibited insulin stimulated glucose uptake and oxidation, which was coupled to decreased expression of GLUT4.

All of these studies reveal that the relationship between CLA and insulin resistance is uncertain and may be species dependent. In ZDF rats, CLA mixtures have improved glucose tolerance and fasting serum insulin, while c9,t11 CLA alone has not. In non-diabetic and *ob/ob* mice, CLA mixtures have increased insulin resistance, with the t10,c12-CLA isomer implicated as the major effector.

CLA and Skeletal Muscle

Skeletal muscle is the primary site of insulin-stimulated glucose uptake (Houseknecht & Kahn 1997), and thus, is a possible target for CLA action. In most models of insulin resistance or diabetes, skeletal muscle glucose transport is not limited by GLUT4 expression but is attributable to defects in GLUT4 translocation (Houseknecht & Kahn 1997).

As previously stated in an earlier section, CLA significantly improves impaired glucose tolerance in ZDF rats and the antidiabetic effects are mediated by specific CLA isoforms (Ryder *et al.*, 2001). Consistent with improved glucose

tolerance, it has been show that insulin-stimulated glucose transport was improved in soleus muscle, and insulin-stimulated glycogen synthase activity was improved in soleus and EDL muscles of ZDF rats fed a 50:50 (47.0%c 9,t11 and 47.9% t10,c12 isomers) CLA mixture. The fact that these effects were not observed in rats fed a c9,t11 CLA diet suggests that the effects are specific to the t10,c12 CLA isomer (Ryder *et al.*, 2001). Despite finding improved whole-body glucose tolerance and improved insulin action in skeletal muscle of ZDF rats fed the 50:50 CLA diet (Ryder *et al.*, 2001), it was also observed that there was no effect of the 50:50 CLA diet on insulin signaling in skeletal muscle measured by quantifying PI3K and Akt activities in EDL and soleus muscles in the absence and presence of insulin. It was proposed that the insulin-sensitizing effects of CLA on skeletal muscle may be secondary to reduced adiposity because visceral adiposity is correlated with reduced insulin action in skeletal muscle and liver.

Because skeletal muscle is a major site of insulin-stimulated glucose uptake (Houseknecht & Kahn 1997), skeletal muscle is a major target for therapeutic intervention. It has been suggested that insulin sensitivity may be related to the fatty acid composition of the phospholipids within muscle membranes associated with insulin action (Henriksen *et al.*, 2003). Consumption of CLA has been reported to increase the CLA content of skeletal muscle, adipose tissue, and plasma in several species including humans. Thus, CLA uptake into insulin target tissues may have an impact on insulin action in those tissues (Henriksen *et al.*, 2003). Henriksen *et al.* (2003) studied female obese

Zucker *fa/fa* rats fed either a 50:50 (c9,t11, and t10,c12) CLA mixture, a c9,t11 CLA isomer, or a t10,c12 CLA isomer. Henriksen *et al.* (2003) found that a 50:50 CLA mixture as well as the t10,c12 CLA isomer lead to a significant enhancement of oral glucose tolerance that was associated with enhanced insulin-mediated glucose transport activity assessed by 2-deoxyglucose (2-DG) uptake, determined in the absence or presence of a maximally effective concentration of insulin in both type I and type IIb fibre skeletal muscle. However, the c9,t11 CLA isomer had no effect on glucose tolerance or insulin-mediated glucose transport activity. The authors concluded that these metabolic improvements resulting from CLA treatment can be ascribed specifically to the t10,c12 CLA isomer, with the c9,t11 CLA isomers being nearly metabolically neutral in this animal model of insulin resistance.

Thus, improvements in insulin action have been associated with the t10,c12 CLAisomer and, therefore, diets enriched in the t10,c12 CLA isomer may be efficacious in the management of obesity and insulin resistance.

CLA and TNF-α

Human obesity is positively correlated to TNF-α expression in adipose and muscle tissue (Hotamisligil *et al.*, 1995; Nilsson *et al.*, 1998), and plasma levels of TNF-α are positively associated to body mass index, fasting glucose and serum triglycerides. TNF-α is inversely related to high-density lipoprotein

cholesterol (Nilsson *et al.*, 1998). Thus, TNF-α levels are associated with characteristics of the metabolic syndrome and it has further been suggested that TNF-α is responsible for the pathogenesis of Type 2 diabetes mellitus and insulin resistance (Nilsson *et al.*, 1998) and thus, is a possible target for CLA action.

In one study by Smedman (2005), fifty-three healthy women and men aged 23 to 63 years were randomly assigned to either a CLA mixture group or a control group. Subjects were given control capsules containing olive oil during the initial 2 weeks. For the next 12 weeks, the subjects in the CLA-group were given capsules containing 4.2 g per day of CLA and the control group continued taking control capsules containing the corresponding amount of olive oil. The CLA capsules contained 75.9% CLA with equal amounts of the CLA isomers c9,t11 and t10,c12, respectively, and only minor amounts of other isomers. At baseline, TNF-α did not correlate with any measure of obesity. CLA supplementation did not have any significant effects on serum TNF-α levels, even though there was an increase in plasma C-reactive protein, an inflammatory marker associated with measures of obesity.

In another study by Tsuboyama-Kasaoka *et al.* (2000), 8 week old female C57BL/6J mice were fed a standard semipurified diet (10% fat of total energy) with or without CLA (1% wt/wt) for 4 days to 8 months. CLA was prepared as a free fatty acid with a mixture of isomers (34% c9, t11/t9, and c11; 36% t10 and

c12; 3% c9, c11/c10, and c12; 2% t9, t11/t10, and t12 from total fatty acids). It was found that TNF-α mRNA levels in white adipose tissue increased 8-fold in CLA-fed mice, whereas those in gastrocnemius muscles decreased. Insulin tolerance showed noticeable insulin resistance in CLA fed mice. Although there was no difference in body weight, CLA fed mice showed a marked reduction in brown adipose tissue and a marked decrease of subcutaneous white adipose tissue. They also showed that both cell death and decreased cell size contributed to the decrease in white adipose tissue and brown adipose tissue mass. The development of insulin resistance was shown in CLA fed mice, where increases of fasting and feeding insulin concentrations by 4-fold and 8-fold, respectively, were seen.

These studies support the idea that increased expression of TNF-α (depending on the tissues and species) may induce body fat decrease, apoptosis and insulin resistance. Although TNF-α may be a mediator of obesity-linked insulin resistance and has been proven to elicit insulin resistance in adipose tissue, it is still inconclusive as to whether the same effect is seen in skeletal muscle tissue, and whether CLA reduces TNF-α levels, particularly in skeletal muscle. Also, expression of TNF-α by skeletal muscle still remains controversial.

CLA and AMPK

No studies have looked at the direct effects of CLA supplementation on the levels of AMPK. Since, AMPK has been shown to mediate the action of antidiabetic drugs such as metformin and rosiglitazone (Musi *et al.*, 2002; Fryer *et al.*, 2002), it is plausible that AMPK activators could be developed as antidiabetic agents. CLA has been shown to enhance adiponectin levels in adipose tissue as well as plasma in the Zucker *fa/fa* rat model (Nagao *et al.*, 2003; Noto *et al.*, 2004,2005; Zirk *et al.*, 2005), and plasma adiponectin was recently shown to activate AMPK-α within skeletal muscle (Tomas *et al.*, 2004). Therefore, CLA may improve insulin sensitivity via skeletal muscle AMPK-α, through the increase of plasma adiponectin levels.

CLA in Human Studies

The effects of CLA on human metabolism are not as established as those in animal models. One example of this is the Netherlands Cohort Study by Voorrips *et al.* (2002) where it was found that there was no evidence for a protective effect of CLA on breast cancer incidence in postmenopausal women, as was suggested by animal experiments. The absence of a relationship between breast cancer incidence and intake of energy or fat was in line with current opinion.

Some studies have shown that CLA mixture supplementation in healthy people had no effect on serum glucose (Smedman & Vessby, 2001) and serum insulin (Medina *et al.*, 2000).

Other studies have reported negative effects. Riserus *et al.* (2002) used a randomized, double blind controlled trial on abdominally obese men (n = 60) to investigate treatment with 3.4 g/day of either a CLA mixture, purified t10,c12 CLA, or a placebo. The results showed that the t10,c12 CLA isomer, but not a CLA mixture, significantly increased insulin resistance, fasting glucose, and dyslipdemia in abdominally obese men. This lead to the conclusion that the t10,c12 CLA might be diabetogenic in the metabolic syndrome. This is in agreement with findings from CLA-fed mice, but unlike the findings in ZDF rats, suggesting important isomer-specific metabolic effects of CLA that are species dependent.

Another study by Riserus *et al.* (2002) showed that t10,c12 CLA supplementation increases oxidative stress and inflammatory biomarkers in obese men. Oxidative stress was concluded to be closely related to induced insulin resistance, suggesting a link between the fatty acid-induced lipid peroxidation and insulin resistance.

In terms of body composition, Mougios *et al.* (2001) found that the CLA mixture significantly reduced the sum of 10 skin folds and percent body fat

between weeks 4 and 8 of supplementation in overweight subjects. Blankson *et al.* (2000) found that when overweight or obese human subjects were supplemented with CLA mixture (3.4-6.0 g/day) for 12 weeks, a significant reduction of fat mass was observed. However, Zambell *et al.* (2000) found there was no benefit in people consuming 3.0 g/day CLA mixture for 12 weeks, with respect to body weight or adiposity.

Thus, the effects of CLA on insulin sensitivity and body composition in humans are not conclusive. There is some indication that t10,c12 CLA may have negative effects on insulin sensitivity in the metabolic syndrome.

Zucker (*fa/fa*) Rat Model

Rodent models of disease are very useful for studying disease pathophysiology and also for testing safety and effectiveness of potential treatments. Lois and Thiodor Zucker first described the obese *fa/fa* Zucker rat in 1961. These rats carried an autosomal recessive *fa* gene for obesity that leads a deficiency in the leptin receptors causing hyperphagia and obesity. This causes insulin resistance, hyperinsulinemia and glucose intolerance. Now it has been shown that *fa/fa* Zucker rats also have reduced sympathetic activity to brown adipose tissue, reduced heat production, increased glucose utilization of white adipose tissue, increased de novo fatty acid synthesis, elevated serum

triglycerides, increased lipoprotein lipase activity of white adipose tissue and elevated serum corticosterone concentration (White & Martin, 1997).

Terrettaz *et al.* (1983) used the techniques of hyperglycemic and euglycemic clamps adapted to small rodents to measure *in vivo* peripheral (muscle, adipose tissues) glucose metabolism and *in vivo* hepatic glucose production, in lean and genetically obese (*fa/fa*) rats. It was observed that during either hyperglycemic or euglycemic clamps, peripheral glucose metabolism by muscle and adipose tissue of obese rats was similar to that of lean controls but at the cost, as the obese rats had plasma insulin levels that were 3.5-fold higher than control. This result indicated that peripheral tissues of obese rats were indeed insulin resistant when tested *in vivo*. It was also observed that raising plasma insulin levels in lean rats inhibited *in vivo* hepatic glucose production. In contrast, in obese rats, hepatic glucose production was high in spite of a marked increase in basal insulinemia. Furthermore, hepatic glucose production of obese rats failed to be inhibited by further increasing their hyperinsulinemia.

Crist *et al.* (1998) showed that the marked reduction in whole body glucose uptake in the *fa/fa* Zucker rat is due to the decreased ability of insulin to stimulate glucose uptake in skeletal muscle, but not in adipose tissue. Moreover, in the obese animal, activation of A_1 adenosine receptors (A_1AR) within skeletal muscle has been shown to lower insulin sensitivity, thereby increasing the concentration of insulin needed to stimulate glucose and amino acid transport

into muscle. Antagonism of A_1AR was accomplished using the A_1AR antagonist, 1,3-dipropyl-8-(p-acrylic) phenylxanthine (BWA1433), which does not penetrate the blood–brain barrier and was consequently able to improve whole body glucose disposal by selectively increasing glucose uptake in skeletal muscle. These data support the conclusion that insulin resistance in the *fa/fa* Zucker rat is tissue specific.

The cause of insulin resistance in the *fa/fa* Zucker rat has yet to be elucidated. Liu *et al.* (2002) compared plasma concentrations of leptin, free fatty acids and TNF-α in *fa/fa* and lean Zucker rats. The *fa/fa* but not the lean rats had significantly elevated concentrations of all three circulating proteins when compared at 6 and 15 weeks of age. Free fatty acid concentrations were much higher than TNF-α at 6 weeks of age. Thus, it was concluded that free fatty acids play a greater role in insulin resistance up to 6 weeks of age. In contrast, free fatty acids along with TNF-α act together to elevate insulin resistance after 6 weeks of age. Elevated expressions of GLUT4 (2x), fatty acid synthase (6x) and acetyl-coA carboxylase (15x) in white adipose tissue and glucokinase (3x) in the liver are associated with hyperinsulinemia in the *fa/fa* Zucker rats (Assimacopoulos-Jeannet, 1995).

2. STUDY RATIONALE

Limitations of Published Research

Although numerous publications have provided favorable results regarding the effects of CLA isomers and CLA mixtures, there are many inconsistencies in the literature. Contrary results have been seen with the use of different species, variation of dose, duration of CLA supplementation, and composition of CLA isomers. Different animal models have shown varying degrees of sensitivity to CLA and CLA mixtures. Pioneering research with regards to CLA did not always define which CLA isomers were used, creating difficulty in ascertaining the true CLA isomer(s) responsible for the biological effects. It has been shown that individual CLA isomers such as c9,t11 CLA and t10,c12 CLA have different effects in different species, and their mechanisms of action are not clearly known.

Ryder *et al.* (2001) showed that feeding a blend of the two CLA isomers (t10,c12 and c9,t11) improved lipid and glucose metabolism in ZDF rats. They showed that the c9,t11 CLA isomer alone improved TAG metabolism. In contrast, when Tsuboyama-Kasaoka *et al.* (2000) fed normal weight C57BL/6J mice a similar blend of CLA isomers, the diet induced a lipoatrophic diabetic state. Roche *et al.* (2002) showed that the effect shown by Ryder *et al.* (2001) may be attributed to the t10,c12 CLA isomer. Roche *et al.* (2002) also suggested that it

is probable that the divergent effects of CLA on insulin and glucose metabolism that had been reported by Ryder *et al.* (2001), and Tsuboyama-Kasaoka *et al.* (2000) reflect the different metabolic consequences of the antiobesity effect of t10,c12 CLA between animal models. In the ZDF rat, hyperglycemia is secondary to obesity; therefore, the antiobesity action of t10,c12 CLA would have partly accounted for improved glucose metabolism. However, in the C57BL/6J mouse, the antiobesity action of t10,c12 CLA resulted in lipodystrophy that probably induced the prodiabetic state. This shows that there are many variances from study to study depending on the research design, the species, and supplementation protocol.

When examining AMPK, limitations of previous research are also apparent. Evidence in support of a role for AMPK in contraction-stimulated glucose transport came from studies using AICAR. AICAR is used to activate AMPK in skeletal muscle. These studies showed that AMPK is sufficient to stimulate glucose uptake and that AMPK-mediated glucose uptake involves glucose transporter translocation (Fryer *et al.*, 2002). Nevertheless, AICAR is not selective for AMPK. Furthermore, treatment with alleged AMPK inhibitors, which are not specific to AMPK, will inhibit AICAR-induced activation of AMPK, but will not inhibit contraction-induced AMPK activation in skeletal muscle (Fryer *et al.*, 2002). These results suggest that there may be other mechanisms in addition to AICAR-stimulated AMPK activation and imply that one or more additional pathways are involved in mediating glucose transport in skeletal muscle.

Another limitation associated with AMPK is related to its link with fatty acid oxidation in skeletal muscle by way of ACC. It is known that AMPK can inactivate ACC, and thus block conversion of acetyl-CoA to malonyl-CoA, the first intermediate in the formation of long-chain fatty acids. It is not clear, however, if AMPK is important *in vivo*, since ACC phosphorylation can also be regulated hormonally by epinephrine and glucagon via other protein kinases.

TNF-α research also has its limitations. Although there is a body of evidence showing that TNF-α reduces insulin-stimulated glucose uptake and also increases lipolysis in adipose tissue (Sethi & Hotamisligil, 1999), there is less convincing evidence to support a causal relationship between TNF-α and insulin resistance in skeletal muscle. Since reduced insulin-mediated glucose uptake in skeletal muscle is a cardinal feature of insulin resistance in Type 2 diabetes (Manco *et al.*, 2000), it is important to determine whether TNF-α causes insulin resistance in skeletal muscle.

TNF-α is expressed in adipocytes and induces insulin resistance in adipocytes (Sethi & Hotamisligil, 1999). On the other hand, both acute and prolonged treatment of skeletal muscle with TNF-α can increase glucose uptake in some situations (Ciaraldi *et al.*, 1998). Production of TNF-α may therefore be a compensatory mechanism for reduced insulin-stimulated glucose uptake that is seen in Type 2 diabetes.

CLA, AMPK and TNF-α have all been shown to correlate with insulin sensitivity and hyperlipidemia, however, their effects and mechanisms within skeletal muscles have yet to be fully determined. Although many mechanisms have been proposed, further experimental research is required.

Study Rationale

A critical defect in the development of glucose intolerance and Type 2 diabetes is insulin resistance and reduced skeletal muscle glucose disposal (Manco *et al.*, 2000). Insulin resistance and compensatory hyperinsulinemia are closely associated with several atherogenic risk factors, such as hypertension, hyperlipidemia, and central obesity, and this condition is referred to as "Syndrome X" or the "insulin resistance syndrome" (Shulman, 2000). It is therefore crucial that strategies be developed to reduce insulin resistance of skeletal muscle glucose transport as a possible means for treating Type 2 diabetes.

Different ways of promoting the use of functional foods and nutraceuticals for the possible prevention and treatment of diseases such as Type 2 diabetes are being used by the media, researchers and supplement companies. The food and drug authorities do not approve many of these products; yet they do not require approval in order to have these items sold to the general public. Most of these products have not even been found to be effective and they may even

produce undesirable side effects, since proper controlled scientific research has not been conducted with regards to safety of these products.

CLA is one functional food and/or nutraceutical that may offer health benefits for many diseases, including Type 2 diabetes. CLA has been researched extensively with respect to many different ailments. Although not all findings are conclusive, it is plausible that CLA isomers and/or mixtures may be beneficial in some of these diseases such as Type 2 diabetes. Some studies suggest that certain CLA isomers and CLA mixtures may have a beneficial effect on insulin resistance in different animal models. CLA has been shown to reduce body fat in growing mice and increase fat mass loss in adult humans (Roche *et al.*, 2002; Blankson *et al.*, 2000). Moreover, CLA treatment has been shown to enhance glucose tolerance and insulin-stimulated glucose transport activity and glycogen synthase activity in skeletal muscle of the Zucker diabetic fatty (ZDF) rat, a rodent model of Type 2 diabetes (Ryder *et al.*, 2001). However, the mechanisms are not fully understood and need to be investigated further to elucidate their function in the human body.

There has not been enough research conducted that has directly compared the individual metabolic effects of treatment with enriched preparations of c9,t11 CLA and t10,c12 CLA, along with various mixtures of CLA isomers on whole body and skeletal muscle insulin action in animal or human models of insulin resistance. One of the possible mechanisms for reducing insulin

resistance with CLA supplementation might be linked with the activation of AMPK protein levels within skeletal muscle. In addition, CLA may reduce TNF-α protein levels within skeletal muscle.

AMPK has been linked with muscle glucose uptake (Fryer *et al.*, 2002). The insulin resistance associated with Type 2 diabetes is most profound at the level of skeletal muscle as this is the primary site of glucose and fatty acid utilization. Therefore, an understanding of how AMPK is activated in skeletal muscle via diet would offer significant benefits in the treatment of Type 2 diabetes.

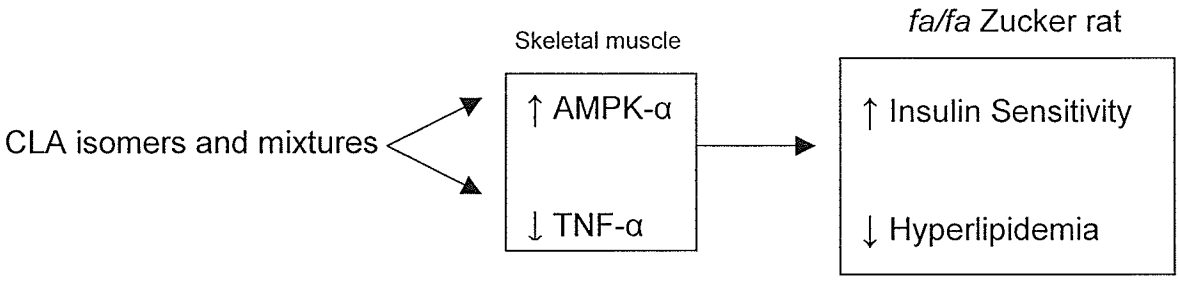
Recent studies have also demonstrated that TNF-α directly interferes with the insulin-signaling cascade at an early step and thus impairs insulin-stimulated glucose transport (Sethi & Hotamisligil, 1999), and that high levels of TNF-α protein are associated with obesity (Uysal *et al.*, 1997). Such findings support the hypothesis that TNF-α might be a mediator of obesity-linked insulin resistance. Conversely, some studies suggest that TNF-α may in fact stimulate glucose uptake in human skeletal muscle cell cultures (Ciaraldi *et al.*, 1998).

Thus, the aim of this study is to investigate the effects of specific CLA isomers on hyperlipidemia, insulin resistance, skeletal muscle AMPK-α, and TNF-α levels in *fa/fa* and lean Zucker Rats.

Hypotheses

1. It is hypothesized that specific CLA isomers such as the t10,c12 CLA isomer, and synergistic effects of a mixture of c9,t11 CLA and t10,c12 CLA isomers, will improve insulin sensitivity and reduce hyperlipidemia in the *fa/fa* Zucker rat.

2. The beneficial effects of the CLA isomers will be mediated through an increase in skeletal muscle AMPK-α levels and activation, and a reduction in TNF-α protein.



Objectives

1. To determine the effects of specific CLA isomers (c9,t11 and t10,c12 individually and in combination) on glycemic control (oral glucose tolerance testing, fasting serum glucose, insulin and C-peptide) and hyperlipidemia (fasting serum free fatty acids and triglycerides) in *fa/fa* and lean Zucker rats.

2. To determine the effect of specific CLA isomers (c9,t11 and t10,c12, individually and in combination) on skeletal muscle AMPK-α levels and phosphorylation, and TNF-α levels using Western immunoblotting.

3. To examine associations between skeletal muscle AMPK-α and TNF-α, glycemic control, and hyperlipidemia.

3. RESEARCH DESIGN & METHODS

Animals and Dietary Treatments

Following a 5-7 day acclimatization period, one hundred and forty, 6 week old male lean and obese *fa/fa* Zucker rats (Harlan Teklad, Madison, WI) were randomly assigned to one of seven dietary treatments for 8 weeks. There were 14 experimental groups (7 dietary groups x 2 genotypes (lean and *fa/fa*)), with n=10 rats per group to ensure an adequate sample size.

All diets were nutritionally sound, based on the AIN-93G diet (Reeves *et al.*, 1993), with fat contributing 8.5% (wt/wt) of the diet. The seven treatments included: 1) 0.4% c9,t11 CLA (9,11) diet (Natural ASA, Hovdebygda, Norway), 2) 0.4% t10,c12 CLA (10,12) diet (Natural ASA, Hovdebygda, Norway), 3) 0.4% c9,t11 CLA plus 0.4% t10,c12 CLA together (TOG) diet (Natural ASA, Hovdebygda, Norway), 4) CLA mixture containing 0.4% c9,t11 CLA isomer plus 0.4% t10,c12 CLA isomer and other CLA isomers (BIO) diet (Bioriginals, Saskatoon, SK), 5) CLA mixture containing 0.4% c9,t11 CLA isomer plus 0.4% t10,c12 CLA isomer plus other CLA isomers (NCK) diet (Nu-Check-Prep, Elysian, MN), 6) Control diet (CTL) and 7) Pair Weighed (PW) group fed the CTL diet in amounts to maintain body weights equivalent to the lightest *fa/fa* or lean Zucker rats, thus ensuring differences in insulin sensitivity were not due to differences in body weight (Tables 1-5).

The commercially available CLA isomer products were in free fatty acid form, although CLA in food is found in triacylglycerol form. The dry ingredients for the diets were pre-mixed and fresh batches of diet containing oil were prepared weekly and stored at -20°C.

Rats were housed individually in a controlled environment of 21-23°C, 55% humidity and a 14:10-h light-dark cycle. Water was administered *ad libitum*. Feed cups were re-filled 3 times weekly, and feed consumption (corrected for spillage) was measured during this time. The pair weighed rats were fed daily when feed restriction was required to control body weight. Weekly body weights were recorded. The University of Manitoba Fort Garry Protocol and Management Committee approved the protocol for the animal care procedures.

Table 1 - Diet Formulation (g/kg)

Ingredients[1]	9,11	10,12	TOG	BIO	NCK	CTL
Dry Mix						
Cornstarch[2]	363	363	363	363	363	363
Maltodextrin	132	132	132	132	132	132
Sucrose	100	100	100	100	100	100
Egg White	212.5	212.5	212.5	212.5	212.5	212.5
Cellulose	50	50	50	50	50	50
Mineral Mix	35	35	35	35	35	35
Vitamin Mix	10	10	10	10	10	10
Choline	2.5	2.5	2.5	2.5	2.5	2.5
Biotin Mix[3]	10	10	10	10	10	10
TBH[4]	0.014	0.014	0.014	0.014	0.014	0.014
Oil[5]						
Soy oil	80.7	80.7	76.3	73	63	85
c9,t11 CLA[6]	4.3	0	4.3	0	0	0
t10,c12 CLA[6]	0	4.3	4.3	0	0	0
BIO[6]	0	0	0	12	0	0
NCK[6]	0	0	0	0	22	0

[1]All ingredients from Harlan Teklad (Madison, WI), unless otherwise indicated

[2]Cornstarch (Best Foods, Etobicoke, ON)

[3]Biotin mix = 200 mg biotin/kg of cornstarch (Egg white was the protein source).

[4]TBH = Tert-butylhydroquinone (Aldrich Chemical Co, Milwaukee, WI)

[5]Total oil in all diets = 85 grams/kg of diet.

[6]See Table 2 for source and composition of CLA

Table 2 - Composition of CLA in Each Diet Treatment

Group	Amount (w/w) and Composition	Source
9,11	0.4% c9,t11 CLA	Natural ASA, Hovdebygda, Norway
10,12	0.4% t10,c12 CLA	Natural ASA, Hovdebygda, Norway
TOG	0.4% c9,t11 CLA and 0.4% t10,c12 CLA	Natural ASA, Hovdebygda, Norway
BIO (CLA 2 isomer mixture)	0.88% CLA mixture containing 0.4% c9,t11 CLA and 0.4% t10,c12 CLA + 0.08% other CLA isomers	Bioriginals, Saskatoon, SK
NCK (CLA 4 isomer mixture)	1.9% CLA mixture containing 0.4% c9,t11 CLA and 0.4% t10,c12 CLA + 1.1% other CLA isomers	Nuchek-Prep, Elysian, MN
Control	8.5% Soybean oil	Harland Teklad, Madison, WI
Pair-Weighed[1]	8.5% Soybean oil	Harland Teklad, Madison, WI

[1]Fed control diet in amounts to maintain body weight equal to the CLA group with the lowest body weight within each of the lean and *fa/fa* genotypes. This was done to ensure that body weight differences would not be responsible for changes in the parameters measured.

Table 3 - Percentage of Fatty Acid Composition in Each Oil

Fatty Acids	9,11	10,12	BIO	NCK
c9,t11 CLA	92.7	4.3	32.9	17.8
t10,c12 CLA	0.6	92.3	33.2	19.5
t8,c10 CLA	0	0	0	18.6
c11,t13 CLA	0	0	0	18.7
Other CLA isomers	2.7[1]	2.4[1]	7.1[1]	12.7[1]
Total CLA Isomers (%)	96	99	73.2	87.3
18:1 c9 (oleic)	4.0	0.8	12.6	N/A
18:2 c9,c12 (linoleic)	<0.1	<0.1	4.1	N/A
Other	0	<0.1	10.1	N/A
Total	100	100	100	100

[1]Actual composition has not been determined.

NA - Not Available.

Table 4 - Total CLA in Each Diet (g/kg)

Diet	c9,t11 CLA	t10,c12 CLA	t8,c10 CLA	c11,t13 CLA	Other CLA Isomers	Total CLA
9,11	3.99	0.03	0	0	0.12	4.13
10,12	0.19	3.97	0	0	0.10	4.26
TOG	4.18	4.00	0	0	0.22	8.39
BIO	3.95	3.98	0	0	0.85	8.78
NCK	3.92	4.28	4.09	4.11	2.79	19.2

Table 5 - Total % CLA in Each Diet

Diet	Total % CLA
9,11	0.41
10,12	0.43
TOG	0.84
BIO	0.88
NCK	1.92

Oral Glucose Tolerance Testing

At 7.5 weeks, oral glucose tolerance testing was utilized as an *in vivo* measure of insulin sensitivity. One week prior to the oral glucose tolerance test, the rats underwent a training session to expose them to the procedures (fasting, restraint for blood collection, ingestion of glucose solution, etc.). On the test day, rats were fasted for 5 hours (8:00-13:00h). Once the baseline blood sample was collected, the glucose solution was presented in a plastic syringe for ingestion. The glucose dose was 1 g/kg body weight (~1ml of a 70% glucose solution). Additional blood samples were collected at t=15, t=30, t=60 minutes post glucose administration. Blood was sampled via the sephanous vein in the hind limb. Blood samples were then stored on ice until centrifuged at 1500 revolutions per minute for 15 minutes at 4°C. The resulting serum layer was stored in aliquots at -80°C until glucose concentrations were determined. Post serum glucose analysis, area under the curve for glucose (AUCg) was calculated by the formula:

$$AUCg = \left[\frac{(t15 + t0)}{2} \times 15\ (min)\right] + \left[\frac{(t30 + t15)}{2} \times 15\ (min)\right] + \left[\frac{(t60 + 30)}{2} \times 30\ (min)\right]$$

Where t0, t15, t30, and t60 are the values in mmoles/L.

Tissue Collection

Following a 12-hour overnight fast, the rats were euthanized by CO_2 asphyxiation and cervical dislocation according to the Canadian Council on

Animal Care Guidelines (Olfert *et al.*, 1993). Each rat was weighed following asphyxiation, and blood was collected following the cervical dislocation. The trunk blood was immediately placed on ice until it was centrifuged to separate the serum fraction. Serum was then stored in aliquots and frozen at -80°C. Dissected gastrocnemius muscles were frozen in liquid nitrogen immediately and stored at -80°C until analyzed.

Insulin Radioimmunoassay (RIA) Kit

Linco's Rat Insulin RIA Kit (LINCO Research, Inc. Cat. # RI-13K, St. Charles, MO. USA) was used to quantify insulin in the serum. This kit uses a fixed concentration of ^{125}I-Insulin labeled tracer antigen incubated with a dilution of antiserum (Guinea Pig anti-Rat Insulin serum) and the amount of tracer bound to antibody is measured. The amount of labeled insulin bound to the antibody is inversely proportional to the concentration of unlabeled insulin in the serum.

This assay was done over a two-day period. On the first day, assay buffer was pipetted into the Non Specific Binding (NSB) tubes and Total Binding tubes. Then purified rat insulin in insulin standard buffer was added in concentrations of 0.1, 0.2, 0.5, 1.0, 2.0, 5.0, and 10.0 ng/ml. Two quality controls were used for reference. Samples were pipetted into the remainder of the tubes in duplicate at dilutions from 1x - 2x for lean rats and 10x for *fa/fa* rats. ^{125}I-Insulin was added to all tubes. Rat anti-Insulin antibody was then added to all of the tubes except for

the Total Count tubes (that only contain ^{125}I-Insulin within them) and NSB tubes. All tubes were vortexed and incubated overnight at 4°C. On day two, 1 ml cold Precipitating Reagent was added to all tubes except for Total Count tubes. Again, all tubes were vortexed and incubated for 20 minutes at 4°C. All tubes except for Total Count tubes were centrifuged at 4°C for approximately 20 minutes until there was a visible pellet. The supernatant of all tubes except for the Total Count tubes was decanted and radioactivity in the tubes was measured with a gamma counter.

The percentage of the total binding for each sample was calculated with the following formula:

$$\%\text{Bound/Total bound} = \frac{(\text{Average counts} - \text{NSB})}{\text{Total Bound}} \times 100$$

Using the standard curve from the assay, serum insulin concentrations in ng/ml were determined using Prism 2.01 Software (Intuitive Software for Science, San Diego, CA) and multiplied by the dilution factor. Values for each sample (ng/ml) were changed into SI values (picomoles/L) using the following formula:

$$\frac{\text{Sample (ng/ml)}}{5800} \times 1000 \times 1000 = \text{Sample (picomoles/L)}$$

C-peptide Radioimmunoassay (RIA) Kit

Linco's Rat C-peptide RIA Kit (LINCO Research, Inc. Cat. # RCP-21K, St. Charles, MO. USA) was used to quantify C-peptide in the serum. This kit uses a fixed concentration of ^{125}I-C-peptide labeled tracer antigen incubated with a dilution of Rat C-peptide antiserum and measures the amount of tracer bound to antibody. The amount of labeled C-peptide bound to the antibody is inversely proportional to the concentration of unlabeled C-peptide in the serum.

This assay was done over a three-day period. On the first day, assay buffer was pipetted into the Non Specific Binding (NSB) tubes, Total Binding tubes, and throughout the rest of the tubes in the assay. Then Purified Recombinant Rat C-peptide in Assay Buffer was added in concentrations of 25, 50, 100, 200, 400, 800, 1600 picomoles/L. Two quality controls were used for reference. Samples were pipetted into the remainder of the tubes in duplicate at dilutions of 10x for lean rats and 40x for *fa/fa* rats. Rat C-peptide Antibody was added to all tubes except for the Total Count tubes and NSB tubes. All tubes were vortexed and then incubated at 4°C overnight. On day two, ^{125}I-Rat C-peptide tracer was added to all of the tubes, followed by vortexing and incubating at 4°C overnight. On day three, cold Precipitating Reagent was added to all tubes except for Total Count tubes. All tubes were vortexed and incubated for 20 minutes at 4°C. All tubes were centrifuged at 4°C for approximately 20 minutes until there was a visible pellet. The supernatant of all tubes except for the Total

Count tubes were decanted and radioactivity in the tubes was measured with a gamma counter.

The percentage of total binding for each sample was calculated with the following formula:

$$\%\text{Bound/Total bound} = \frac{(\text{Average counts} - \text{Non specific binding tubes})}{\text{Total Bound}} \times 100$$

Using the standard curve from the assay, serum C-peptide concentrations in picomoles/L were determined using Prism 2.01 Software (Intuitive Software for Science, San Diego, CA) and multiplied by the dilution factor.

Triglyceride Assay

BioPacific Diagnostics Triglyceride Kit (BioPacific Diagnostics Kit#210, North Vancouver, BC) was used to quantify serum triglycerides. The protocol was adapted for a microplate reader (Spectra Max 340, Molecular Devices Corporation, Sunnyvale, CA, by Gillam, 2003). This procedure includes lipase (Reaction I), glycerol kinase (Reaction II), L-α-glycerol phosphate oxidase (Reaction III) and peroxidase (Reaction IV).

I. Triglycerides $\rightarrow$ Glycerol +Fatty Acids

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

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

IV. 4-Aminoantipyrine + H_2O_2 + DHBS $\rightarrow$ Quinoneimine Dye + HCl + $2H_2O$
(Colorless) (Red color absorbs at 515 nm)

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

Standard concentrations of 0.25, 0.5, 1.0 and 2.0 mmoles/L were prepared by diluting the triglyceride glycerol standard solution (equivalent to 2 mmol triolein) that was provided with the assay kit. The standard concentrations were prepared by diluting the standard solution with 0.9% sodium chloride solution. A quality control was used as reference. A reagent blank (0 mmol) was prepared using 0.9% sodium chloride solution, and serum samples were also diluted with 0.9% sodium chloride to ensure their values fell within the limits of the standard curve. Lean Zucker rat serum samples were not diluted, while *fa/fa* rat samples were diluted 4x, 6x, or 10x depending on the sample subject. All samples and standards were kept on ice. Triplicates of 10 μl aliquots of the prepared standard, blank, quality control and serum samples were pipetted into a 96 well polystyrene plate. Reagent (100 μl) was then pipetted into each well. The plate was incubated at 25°C for 15 minutes. After adding 140 μl deionized water, the absorbance (A) was read at 515 nm (Spectra Max 340, Molecular Devices Corporation, Sunnyvale, California). Measurements were accepted if the standard curve r^2 was greater than 0.9 and the % coefficient of variation (%CV) was less than 10 for the triplicate readings of each standard, blank and

serum sample. Triglyceride concentrations of each serum sample were calculated using the microplate software (SOFTmax Pro, version 1.2.0, Molecular Devices Corporation, Sunnyvale, California) that based the calculation on the standard curve. The concentrations (mmoles/L) were adjusted for dilution factors.

Free Fatty Acid Assay

Roche's Free Fatty Acids, Half-micro test (Roche Diagnostics, Cat. # 1 383 175, Penzberg, Germany) was used to quantify free fatty acids in the serum. The protocol was adapted for a microplate reader (Spectra Max 340, Molecular Devices Corporation, Sunnyvale, CA, by Gillam, 2003). This kit uses reagents to create a red dye that is read at a wavelength of 546 nm to determine free fatty acid levels. In the presence of the enzyme acyl-CoA synthetase (Acyl CS) and ATP, free fatty acids are converted into acyl-CoA, AMP, and pyrophosphate. Acyl-CoA reacts with oxygen in the presence of acyl-CoA oxidase (ACO) to form enoyl-CoA. The resulting hydrogen peroxide (H_2O_2) converts 2,4,6-tribromo-3-hydroxy-benzoic acid (TBHB) and 4-aminoantipyrine (4-AA) to a red dye in the presence of peroxidase (POD) (www.roche-applied-science.com/packinsert /11383175001a.pdf). The dye is measured at 546 nm.

The following chemical formulas show the reactions:

I. Free fatty acids + CoA + ATP $\xrightarrow{\text{Acyl CS}}$ acyl-CoA + AMP + pyrophosphate

II. Acyl-CoA + O_2 $\xrightarrow{\text{ACO}}$ enoyl-CoA + H_2O_2

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

The procedure used 0.200 ml of reaction mixture (A) (containing ATP, coenzyme A, acyl-CoA-synthesase, peroxidase, ascorbate oxidase, 4-aminoantipyrine, potassium phosphate buffer, tribromohydroxybenzoic acid, magnesium chloride and stabilizers) pipetted into each well of a polystyrene 96 well plate. Aliquots of 0.010 ml of double deionized water (blank), serum control sample or experimental sample were pipetted in triplicate into the 96 well plate. The 96 well plate was then placed into the microplate reader and mixed for 30 seconds before being incubated at room temperature (22°C -23°C) for 10 minutes.

Aliquots of 0.010 ml of N-ethyl-maleinimide-solution were added to each well, and the plate was placed back in the microplate reader, mixed for 30 seconds and absorbances (A_1) were read at 546 nm. Following this, 0.010 ml aliquots of reaction mixture B (contained acyl-CoA-oxidase, acyl-CoA-oxidase dilution solution and stabilizers) were added to each well. The 96 well plate was

placed back into the microplate reader, mixed for 30 seconds and incubated at room temperature. After 20 minutes, the 96 well plate was mixed for another 30 seconds and absorbances (A_2) were read at 546 nm.

Calculations for serum free fatty acid concentrations were:

$$C = \frac{V}{\varepsilon \times d \times v} \times \Delta A \text{ [mmoles/L sample solution]}$$

Where:

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

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

d = light path (cm), (0.53326 cm)

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

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

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

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

Free fatty acid concentrations were calculated using the formula:

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

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

Preparation of Skeletal Muscle Protein Samples

Gastrocnemius muscles from each rat were frozen in liquid nitrogen immediately after dissection and stored at -80°C. Samples were taken out of the freezer and immediately stored on ice to avoid thawing. A portion of muscle weighing 0.05 g was taken and placed into a mortar. Liquid nitrogen was then poured over the sample and the muscle sample gently crushed into fine powder with a pestle. Prepared 3x sample buffer (1.5 ml of 30% glycerol, 2% SDS, 0.05 M Tris-HCl pH 6.8) was pipetted over the top of the crushed muscle and mixed together. The sample was then incubated for 15 min at room temperature, while mixing occasionally. It was centrifuged at room temperature for approximately 5 minutes at 13000 revolutions per minute or until separation of protein occurred. The supernatant was then sonicated to assure no solid particles were still present. Samples were stored at -80°C until needed for Western Blotting.

Western Immunoblotting

Bicinchoninic Acid (BCA) protein assays from Pierce (Rockford, IL) were conducted on all samples extracted from gastrocnemius muscle to determine the volume required to load equal amounts of protein (between 20 µg and 30 µg) onto SDS polyacrylamide gels (7.5% gels forAMPK-α [62kDa] or 15% gels for TNF-α [19kDa]). The calculations resulted in volumes of approximately 10 µl-20 µl sample/well.

Protein samples, boiled in a microwave for 3 minutes after the addition of Bromophenol blue (1 µL) and β-mercaptoethanol (1 µL), were loaded onto the stacking gel, and electrophoresis was conducted at 20 mA per gel for 70-90 minutes depending on the percentage of the gel. A control sample (from a PW animal) and prestained markers were loaded on every gel. The PW sample was used as an internal standard for variability in protein loading and band intensity.

Proteins from the gels were transferred onto polyvinylidene fluoride (PVDF) membranes at 100 volts for 60 minutes for 1.0 mm thick gels. PVDF membranes were stored in 1x Tris-buffered saline with Tween-20 (TBST), which is made up of 1 M Tris, pH 7.4 (200 ml), 5 M NaCl (300 ml), Tween-20 (5 ml), 1495 ml dH_2O, creating a 5x TBST. This was then diluted with 4 parts dH_2O and kept at 4°C until antibody treated.

Membranes were blocked with 10 ml of 3% BSA (bovine serum albumin) in TBST for one hour and fifteen minutes at room temperature. Specific primary antibodies were then added at 1:1000 dilution (10 µl primary antibody: 10 ml 3% BSA in TBST) (Table 6) to the membrane and incubated for one hour. Membranes were then washed with 1%TBST for either four washes of 5 minutes (AMPK-α, phospho-AMPK-α) or six washes of 10 minutes (TNF-α). Secondary antibody, which amplifies the signal of the primary antibody, was then added at 1:10,000 dilution (1 µl secondary antibody: 10 ml 1% BSA in TBST) for one hour (Table 6). Membranes were washed in 1% TBST, for four washes of 5 minutes

(AMPK-α, pAMPK-α) or six washes of 10 minutes (TNF-α). The membranes were thoroughly saturated in ECL Western Blotting Detection Reagents (Amersham Biosciences) or ECL plus Western Blotting Detection System (Amersham Biosciences) luminescent reagent and the relative chemiluminescence for each sample was documented by autoradiography.

Protein band intensity (Volume Optical Density (OD*mm^2) - Mean Background OD) was determined with the GS-800 Calibrated Densitometer (Bio-Rad Laboratories, 1-D Analysis Service), and expressed as a ratio of the control sample, which was set to 1.

Table 6 - Antibodies used in Western blotting

Primary Antibody	Name	Reference Source	Source	Dilution Factor	Secondary Antibody	Dilution Factor	Molecular Weight (kDa)
AMPK-α	AMP-activated protein kinase-alpha	Cell Signaling Cat. # 2532	Rabbit	1:1000	Anti-Rabbit Horse Radish Peroxidase	1:10,000	62
P-AMPK-α	Phospho-AMP activated protein kinase-alpha (Thr^{172})	Cell Signaling Cat. # 2531	Rabbit	1:1000	Anti-Rabbit Horse Radish Peroxidase	1:10,000	62
TNF-α[1]	Tumor Necrosis Factor-alpha	Santa Cruz Biotechnology, Inc. Cat. # SC-1352	Goat	1:1000	Anti-Goat Horse Radish Peroxidase	1:10,000	19 (active) 26 (membrane)

[1] TNF-α has a molecular weight of 19 kDa in its active form and a molecular weight of 26 kDa in the bound form in the membrane.

Statistical Analysis

Statistical analysis was carried out using Statistical Analysis System (SAS; The SAS System V9.1 for Windows, SAS Institute Inc., Cary, NC). Results were compiled and analyzed using two-way Analysis of Variance (ANOVA) for the main effects (genotype, diet, and interactions of genotype x diet), and repeated measures Analysis of Variance (ANOVA) was used for OGTT serum glucose concentrations. When a genotype effect or genotype x diet interaction effect was observed, one figure including all groups was presented. When a diet effect was observed an additional figure including the diet means was included. Individual groups were compared using Pre-determined Contrasts. Duncan's Multiple Range test was used for comparisons of OGTT and main diet effects. Trends were considered as a result of Duncan's Multiple Range test. A significant difference was set at a P-value <0.05. All values were reported as means ± standard error of the mean (SEM).

4. RESULTS

Body Weight

The *fa/fa* Zucker rats had a 1.7-fold higher final body weight compared to the lean Zucker rats (547 ± 5 versus 327 ± 3 g, respectively; Figure 1), but there was no effect of dietary CLA treatment. Because dietary CLA treatment did not alter final body weight, the PW groups were excluded from further analysis.

Feed Intake

The *fa/fa* Zucker rats had a 1.6-fold higher total feed intake (1525 ± 20 g) compared to the lean Zucker rats (980 ± 11 g). The *fa/fa* Zucker rats also had a 1.6-fold higher feed intake per day (27.2 ± 0.4 g) compared to the lean Zucker rats (17.5 ± 0.2 g). There was a significant diet x genotype interaction such that *fa/fa* rats fed the 10,12 diet had 16% lower total feed intake and feed intake per day compared to CTL-fed *fa/fa* rats (Figures 2 & 3).

Feed Efficiency Ratio

Feed efficiency ratio (FER) was determined by the body weight gain (g) during the 8 week study divided by the total feed intake (g) for the 8 week study. The *fa/fa* Zucker rats had a 1.2-fold higher FER (0.197 ± 0.003) compared to the

lean Zucker rats (0.157 ± 0.002). There was a significant diet x genotype interaction, such that the *fa/fa* rats fed the TOG diet had 13% higher FER compared to CTL (Figure 4). The *fa/fa* Zucker rats fed the 9,11, BIO and NCK diets had 11%, 7.7%, and 4.8%, respectively, lower FER when compared to the TOG group. There was a trend ($P>0.05$, considered as a result of Duncan's Multiple Range test) for FER to be higher in *fa/fa* rats fed 10,12 diet compared to CTL.

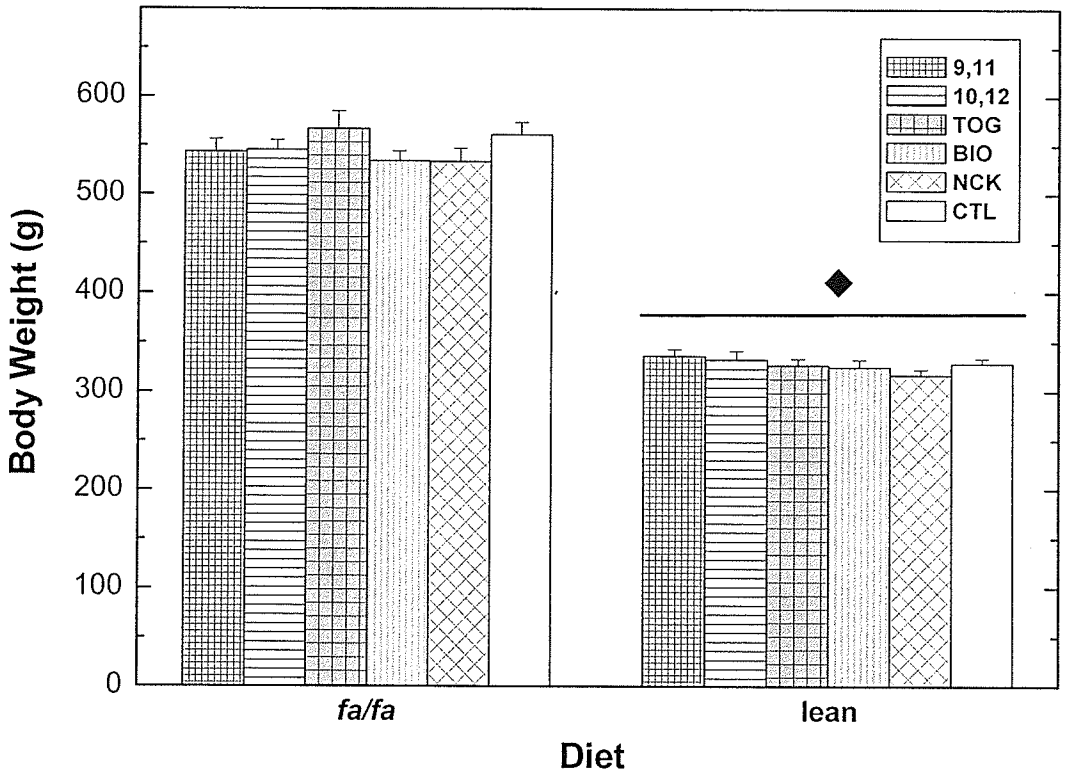


Figure 1 - The effect of 9,11, 10,12, TOG, BIO, NCK and CTL diets on final body weight in Zucker rats. The ◆ symbol denotes a significant genotype effect (P<0.0001). Data are expressed as mean ± SEM (n=10 per group).

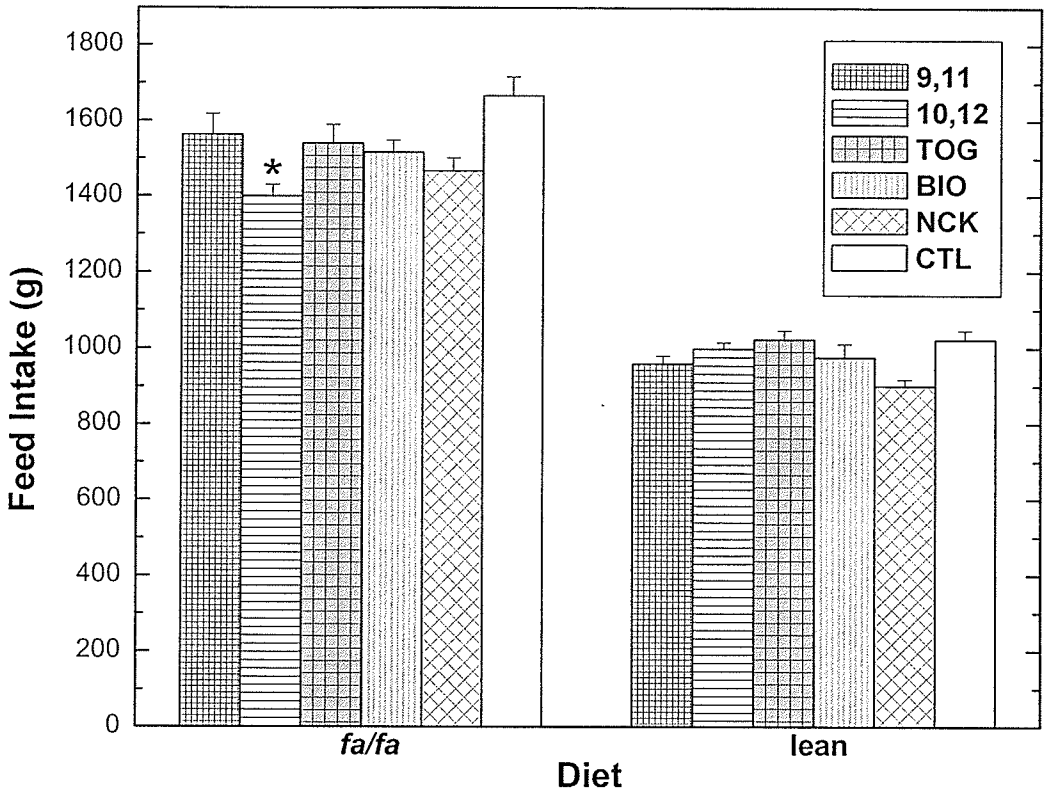


Figure 2 - The effect of 9,11, 10,12, TOG, BIO, NCK and CTL diets on total feed intake in Zucker rats. There was a significant diet x genotype interaction (P=0.0211). The * denotes a significant pre-determined contrast (P<0.05) between a treatment group and CTL. Data are expressed as mean ± SEM (n=10 per group).

Figure 3 - The effect of 9,11, 10,12, TOG, BIO, NCK and CTL diets on feed intake per day in Zucker rats. There was a significant diet x genotype interaction (P=0.0206). The * denotes a significant pre-determined contrast (P<0.05) between a treatment group and CTL. Data are expressed as mean ± SEM (n=10 per group).

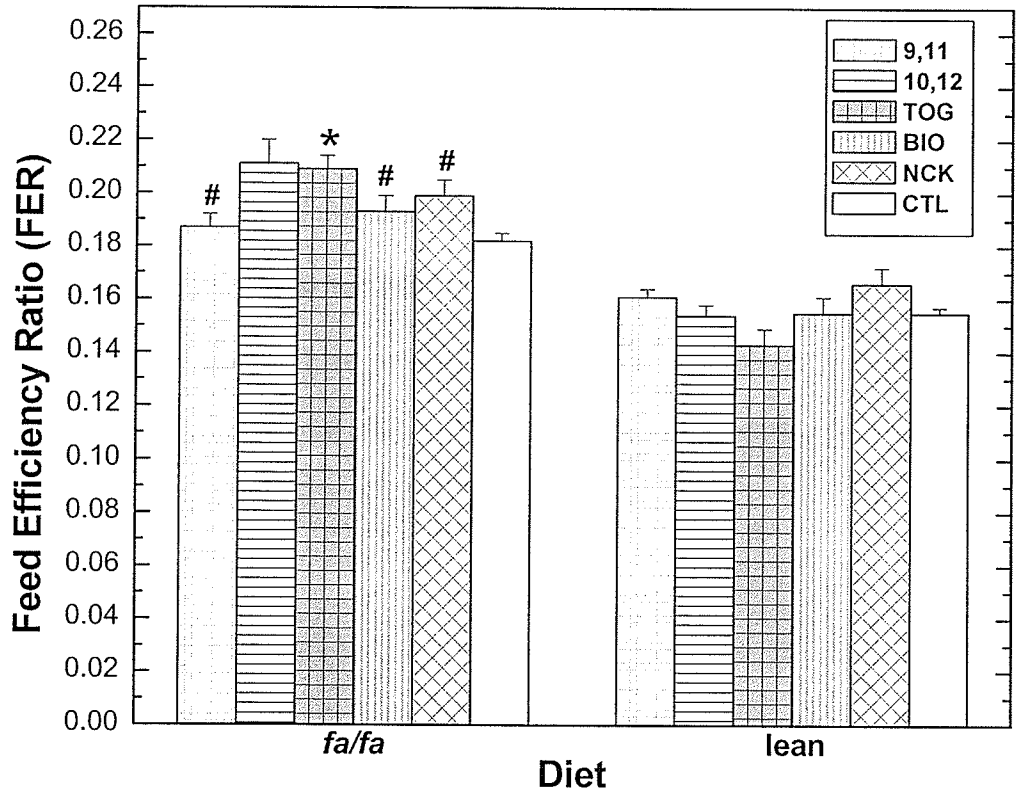


Figure 4 - The effect of 9,11, 10,12, TOG, BIO, NCK and CTL diets on feed efficiency ratio (FER) in Zucker rats. There was a significant diet x genotype interaction (P=0.0008). The * denotes a significant pre-determined contrast (P<0.05) between a treatment group and CTL. The # denotes a significant pre-determined contrast (P<0.05) between a treatment group and TOG. Data are expressed as mean ± SEM (n=10 per group).

Adipose Tissue Mass: Epididymal, Perirenal and Visceral

The *fa/fa* Zucker rats had a 3.6-fold higher epidiymal fat pad weight compared to the lean Zucker rats (19.2 ± 0.4 versus 5.4 ± 0.2 g, respectively; Figure 5). There was a significant CLA diet effect such that Zucker rats fed 10,12, TOG and BIO diets had higher (11-24%) epididymal fat pad weights compared to 9,11 and CTL regardless of genotype (Figure 6). Rats fed NCK diet had 9% lower epididymal fat pad weight compared to TOG regardless of genotype.

The *fa/fa* Zucker rats had a 5.8-fold higher perirenal fat pad weight compared to the lean Zucker rats (27.4 ± 0.7 versus 4.7 ± 0.2 g, respectively; Figure 7), but there was no effect of dietary CLA treatment. There was a trend ($P>0.05$, considered as a result of Duncan's Multiple Range test) for perirenal fat pad weights to be higher in *fa/fa* rats fed 10,12, TOG, BIO, and NCK diets compared to 9,11 and CTL.

Visceral fat pad weight was obtained by adding the epididymal and perirenal fat pad weights. The *fa/fa* Zucker rats had a 4.6-fold higher visceral fat pad weight compared to the lean Zucker rats (46.6 ± 1.0 versus 10.1 ± 0.3 g, respectively; Figure 8). There was a significant CLA diet effect such that Zucker rats fed the 10,12 and TOG diets had 13%-18%, respectively, higher visceral fat pad weights compared to 9,11 and CTL groups regardless of genotype (Figure

9). Rats fed the NCK diet had 4.6% lower visceral fat pad weight compared to the TOG diet regardless of genotype. There was a trend (P>0.05, considered as a result of Duncan's Multiple Range test) for visceral fat pad weights to be higher in *fa/fa* rats fed 10,12, TOG, BIO, and NCK diets compared to 9,11 and CTL.

Adipose Tissue Mass Relative to Body Weight: Epididymal, Perirenal and Visceral

Epididymal, perirenal, and visceral fat pad weights were also expressed relative to body weight (g/100 g) at the end of the 8 week study. The *fa/fa* Zucker rats had a 2.1-fold higher epididymal fat pad weight relative to body weight compared to the lean Zucker rats (3.51 ± 0.06 versus 1.64 ± 0.04 g/100 g, respectively). There was a significant diet x genotype interaction, such that the *fa/fa* rats fed the 9,11 diet had 24% lower epidiymal fat pad weight relative to body weight compared to TOG (Figure 10). There was a trend (P>0.05, considered as a result of Duncan's Multiple Range test) for epididymal fat pad weight relative to body weight to be higher in *fa/fa* rats fed 10,12, TOG, BIO, and NCK diets compared to 9,11 and CTL.

The *fa/fa* Zucker rats had a 3.5-fold higher perirenal fat pad weight relative to body weight compared to the lean Zucker rats (4.98 ± 0.10 versus 1.43 ± 0.05 g/100 g, respectively). There was a significant diet x genotype interaction, such that the *fa/fa* rats fed the TOG, BIO and NCK had 22%, 19% and 22%,

respectively, higher perirenal fat pad weights relative to body weight compared to CTL (Figure 11). The *fa/fa* rats fed the 9,11 diet had 20% lower perirenal fat pad weight relative to body weight compared to TOG.

Visceral fat pad weight relative to body weight was obtained by adding the epididymal and perirenal fat pad weights and expressing them relative to body weight. The *fa/fa* Zucker rats had a 2.8-fold higher visceral fat pad weight relative to body weight compared to the lean Zucker rats (8.49 ± 0.15 versus 3.07 ± 0.08 g/100 g, respectively). There was a significant diet x genotype interaction, such that the *fa/fa* rats fed the TOG, BIO and NCK had 21%, 17% and 20%, respectively, higher visceral fat pad weights relative to body weight compared to CTL (Figure 12). The *fa/fa* rats fed the 9,11 diet had 22% lower visceral fat pad weight relative to body weight compared to TOG.

Adipose Tissue Ratios: Epididymal/Visceral and Perirenal/Visceral

The *fa/fa* Zucker rats had a 1.3-fold lower epididymal/visceral fat pad ratio compared to the lean Zucker rats (0.414 ± 0.005 versus 0.539 ± 0.007, respectively). There was a significant diet x genotype interaction, such that the lean rats fed the NCK diet had a 13% higher epididymal/visceral fat pad ratio compared to CTL (Figure 13).

The *fa/fa* Zucker rats had a 1.3-fold higher perirenal/visceral fat pad ratio compared to the lean Zucker rats (0.586 ± 0.005 versus 0.461 ± 0.007; respectively). There was a significant diet x genotype interaction, such that the lean rats fed the NCK diet had a 15% lower perirenal/visceral fat pad ratio compared to CTL (Figure 14).

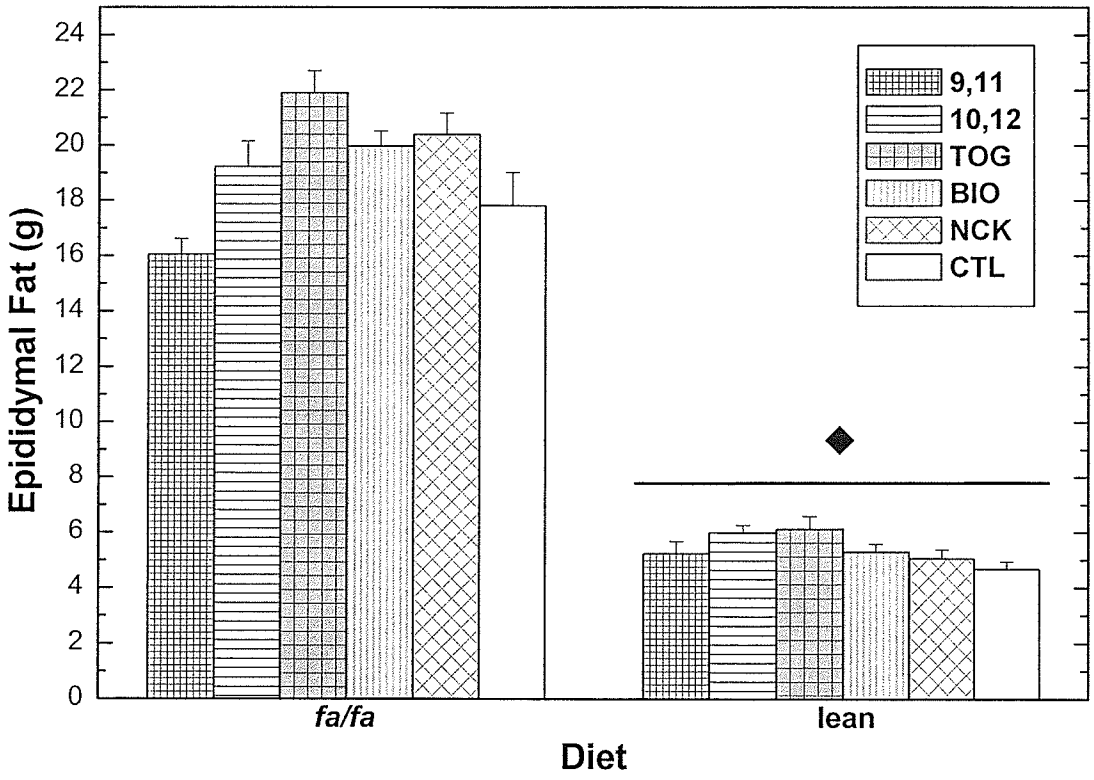


Figure 5 - The effect of 9,11, 10,12, TOG, BIO, NCK and CTL diets on epididymal fat pad weight in Zucker rats. The ◆ symbol denotes a significant genotype effect (P<0.0001). There was a trend (P>0.05, considered as a result of Duncan's Multiple Range test). Data are expressed as mean ± SEM (n=10 per group).

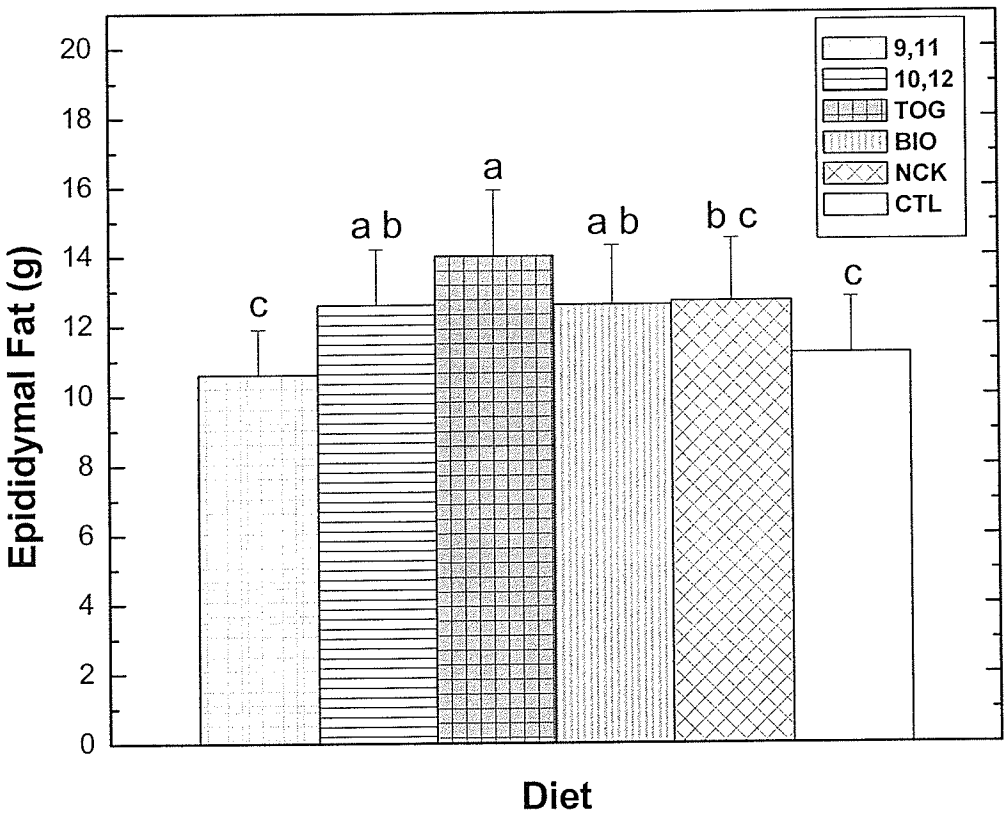


Figure 6 - The effect of 9,11, 10,12, TOG, BIO, NCK and CTL diets on epididymal fat pad weight in Zucker rats. There was a significant diet effect ($P<0.0001$). Means with different letters are significantly different from one another ($P<0.05$), while means with the same letter are not different from one another ($P>0.05$) by Duncan's Multiple Range test. Data are expressed as mean ± SEM (n=20 per group).

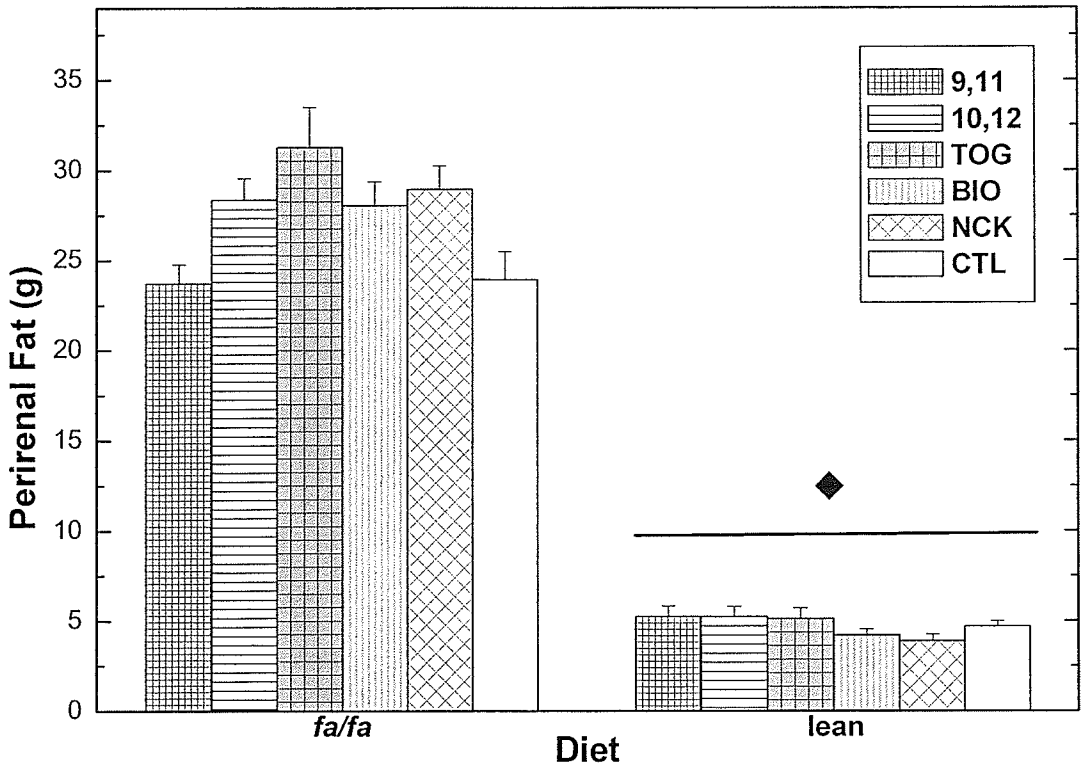


Figure 7 - The effect of 9,11, 10,12, TOG, BIO, NCK and CTL diets on perirenal fat pad weight in Zucker rats. The ◆ symbol denotes a significant genotype effect (P<0.0001). Data are expressed as mean ± SEM (n=10 per group).

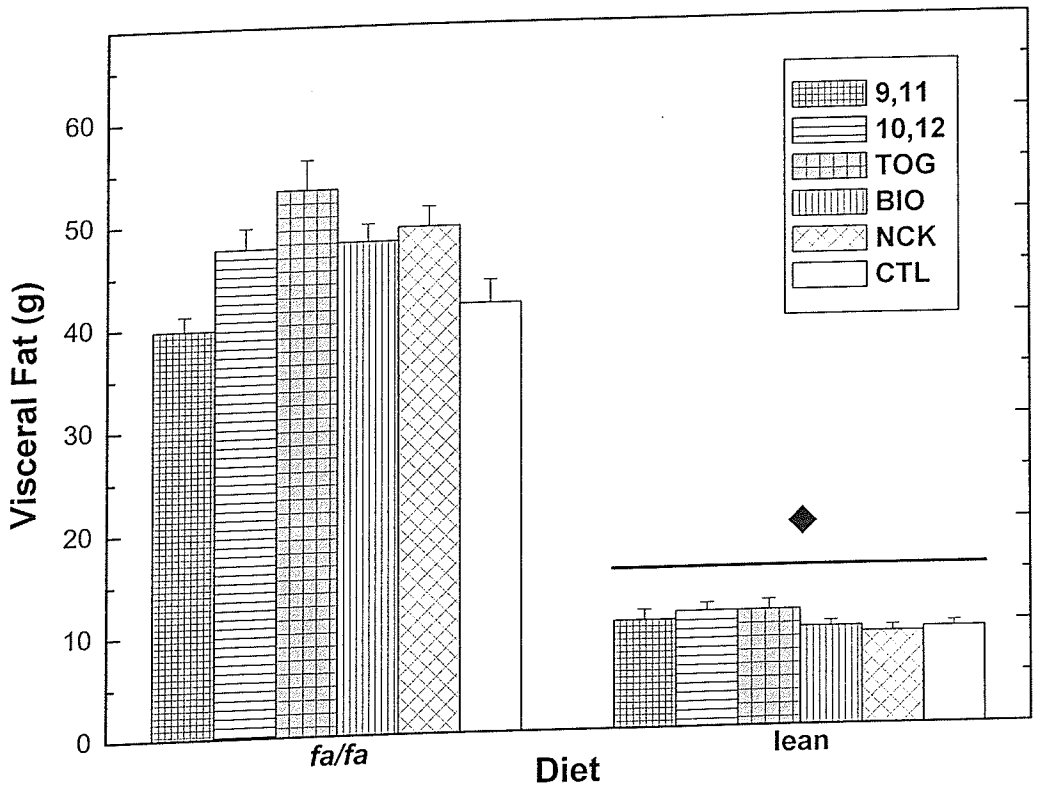


Figure 8 - The effect of 9,11, 10,12, TOG, BIO, NCK and CTL diets on visceral fat pad weight in Zucker rats. The ◆ symbol denotes a significant genotype effect (P<0.0001). Data are expressed as mean ± SEM (n=10 per group).

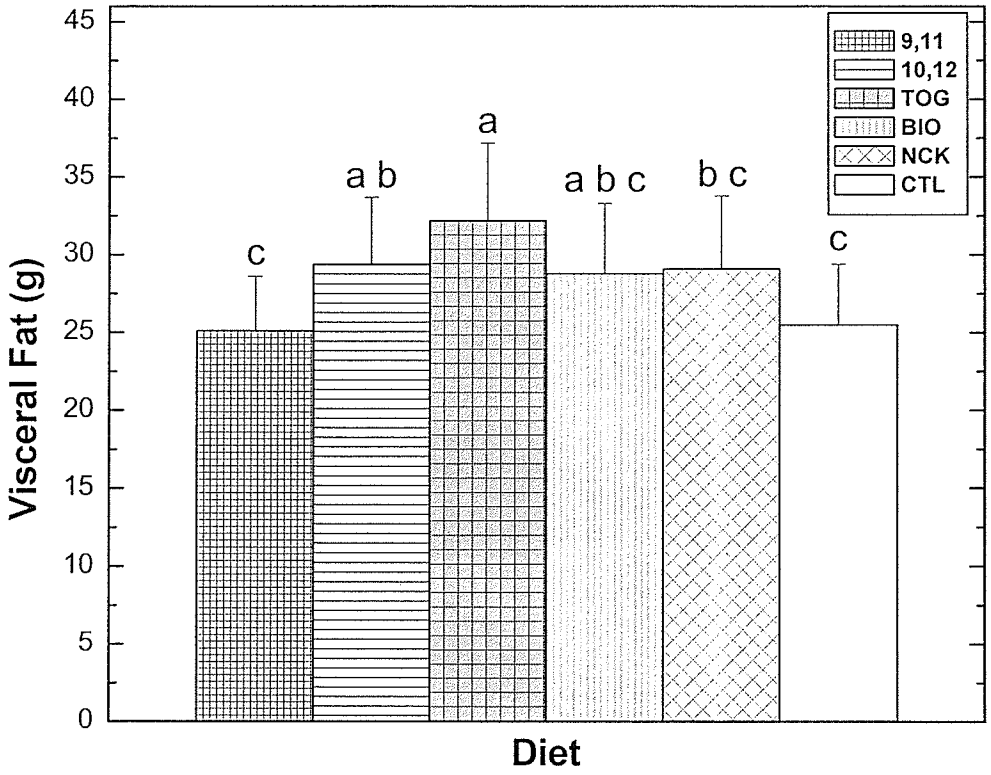


Figure 9 - The effect of 9,11, 10,12, TOG, BIO, NCK and CTL diets on visceral fat pad weight in Zucker rats. There was a significant CLA diet effect (P=0.01). Means with different letters are significantly different from one another (P<0.05), while means with the same letter are not different from one another (P>0.05) by Duncan's Multiple Range test. Data are expressed as mean ± SEM (n=20 per group).

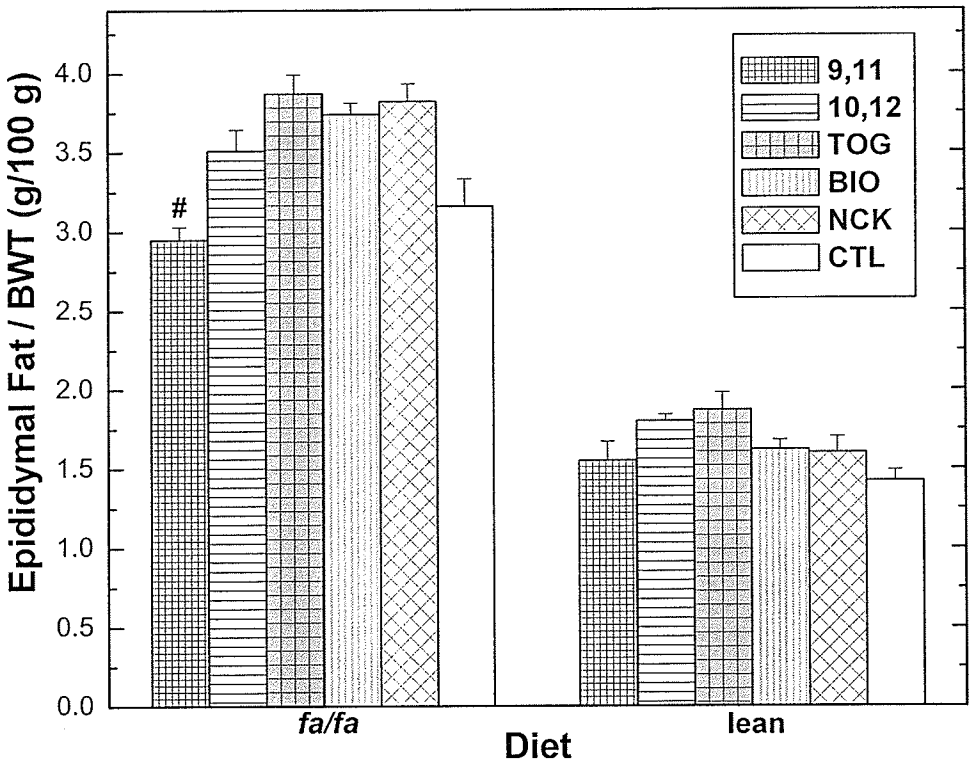


Figure 10 - The effect of 9,11, 10,12, TOG, BIO, NCK and CTL diets on epididymal fat pad weight relative to body weight in Zucker rats. There was a significant diet x genotype interaction (P=0.0013). The # denotes a significant pre-determined contrast (P<0.05) between a treatment group and TOG. Data are expressed as mean ± SEM (n=10 per group).

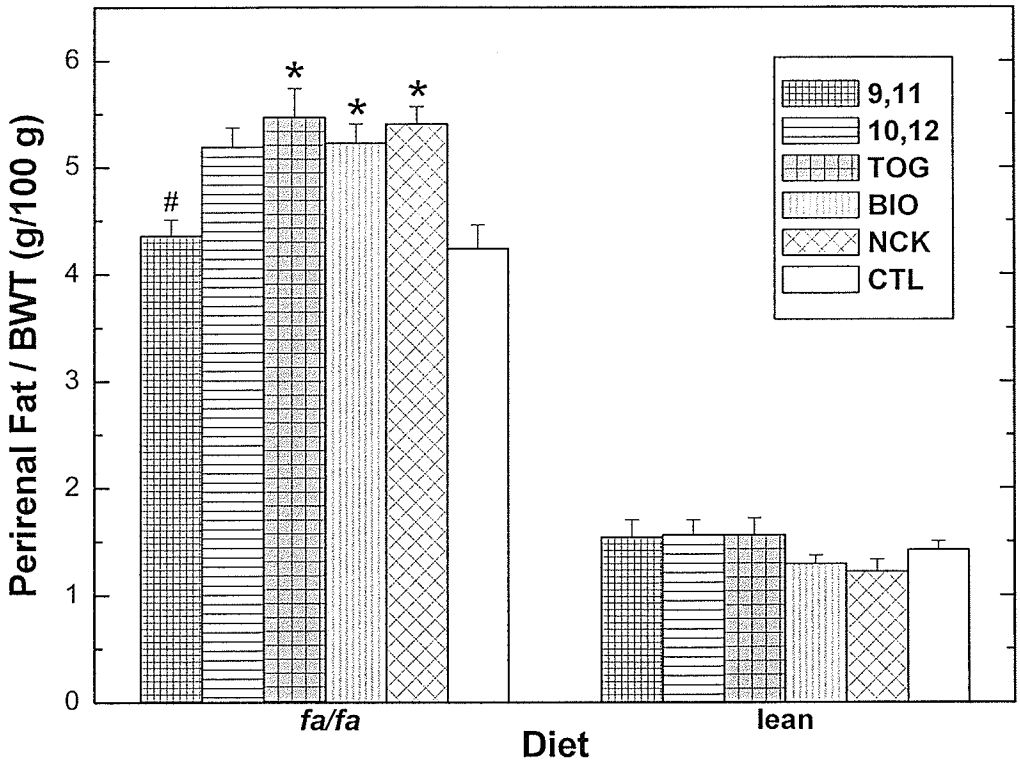


Figure 11 - The effect of 9,11, 10,12, TOG, BIO, NCK and CTL diets on perirenal fat pad weight relative to body weight in Zucker rats. There was a significant diet x genotype interaction ($P<0.0001$). The * denotes a significant pre-determined contrast ($P<0.05$) between a treatment group and CTL. The # denotes a significant pre-determined contrast ($P<0.05$) between a treatment group and TOG. Data are expressed as mean ± SEM (n=10 per group).

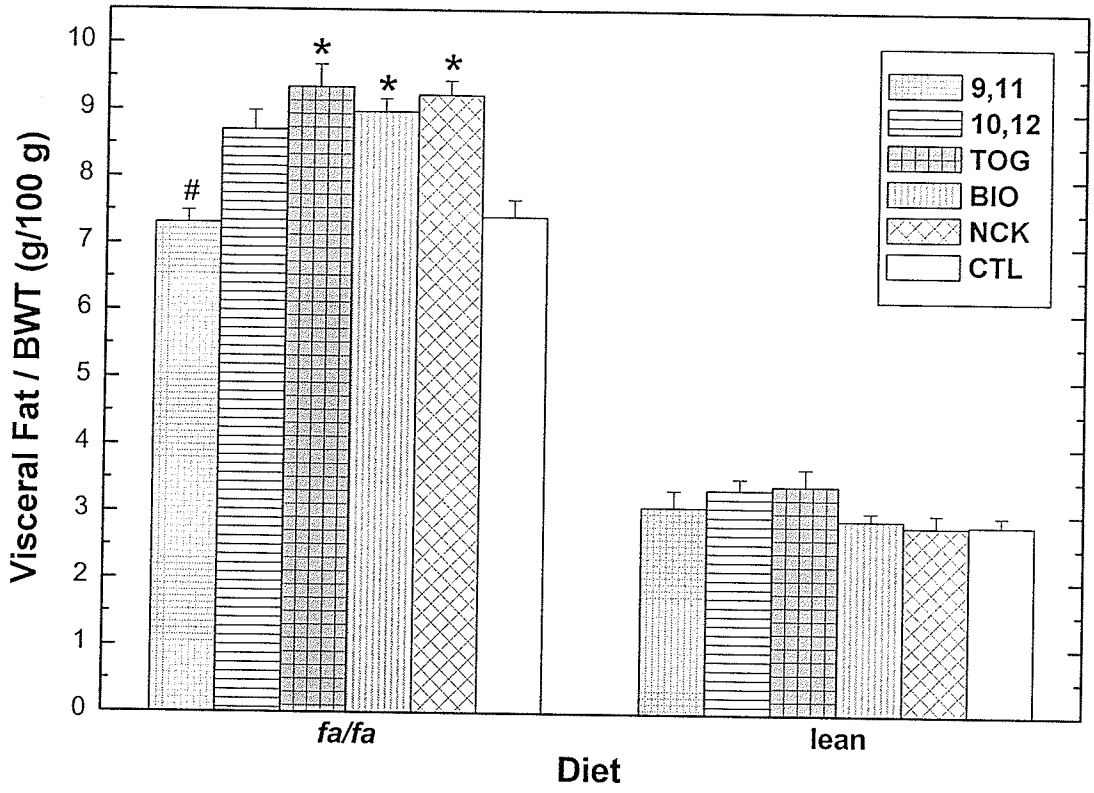


Figure 12 - The effect of 9,11, 10,12, TOG, BIO, NCK and CTL diets on visceral fat pad weight relative to body weight in Zucker rats. There was a significant diet x genotype interaction ($P<0.0001$). The * denotes a significant pre-determined contrast ($P<0.05$) between a treatment group and CTL. The # denotes a significant pre-determined contrast ($P<0.05$) between a treatment group and TOG. Data are expressed as mean ± SEM (n=10 per group).

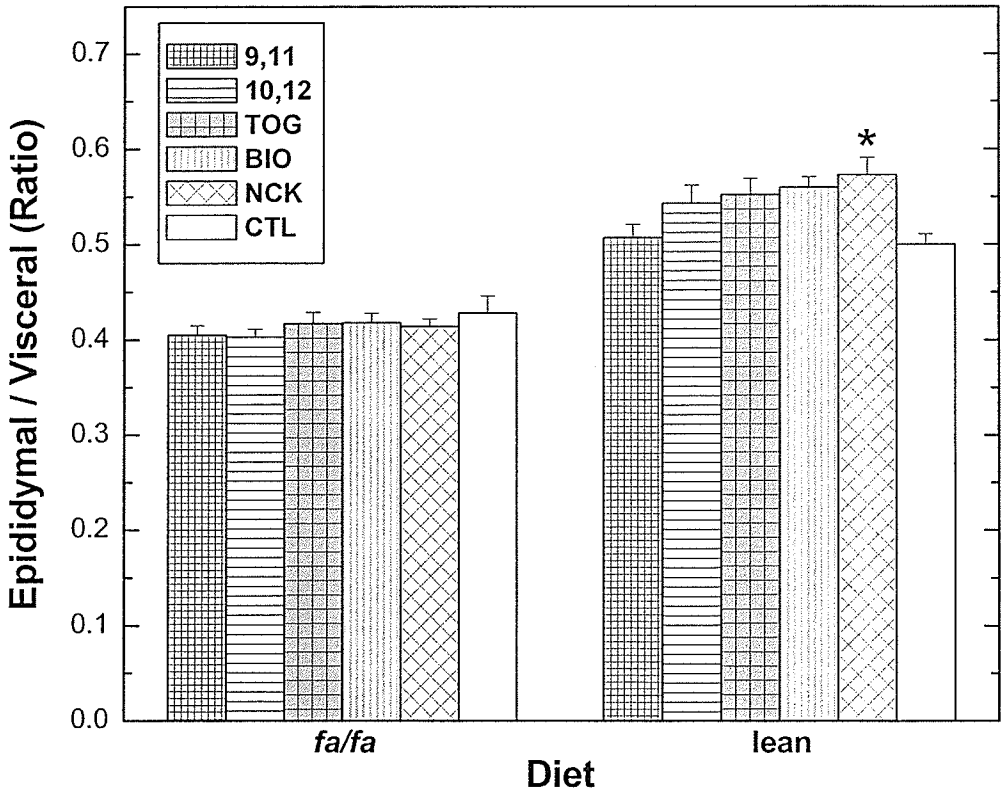


Figure 13 - The effect of 9,11, 10,12, TOG, BIO, NCK and CTL diets on epididymal / visceral fat pad ratio in Zucker rats. There was a significant diet x genotype interaction ($P<0.0232$). The * denotes a significant pre-determined contrast ($P<0.05$) between a treatment group and CTL. Data are expressed as mean ± SEM (n=10 per group).

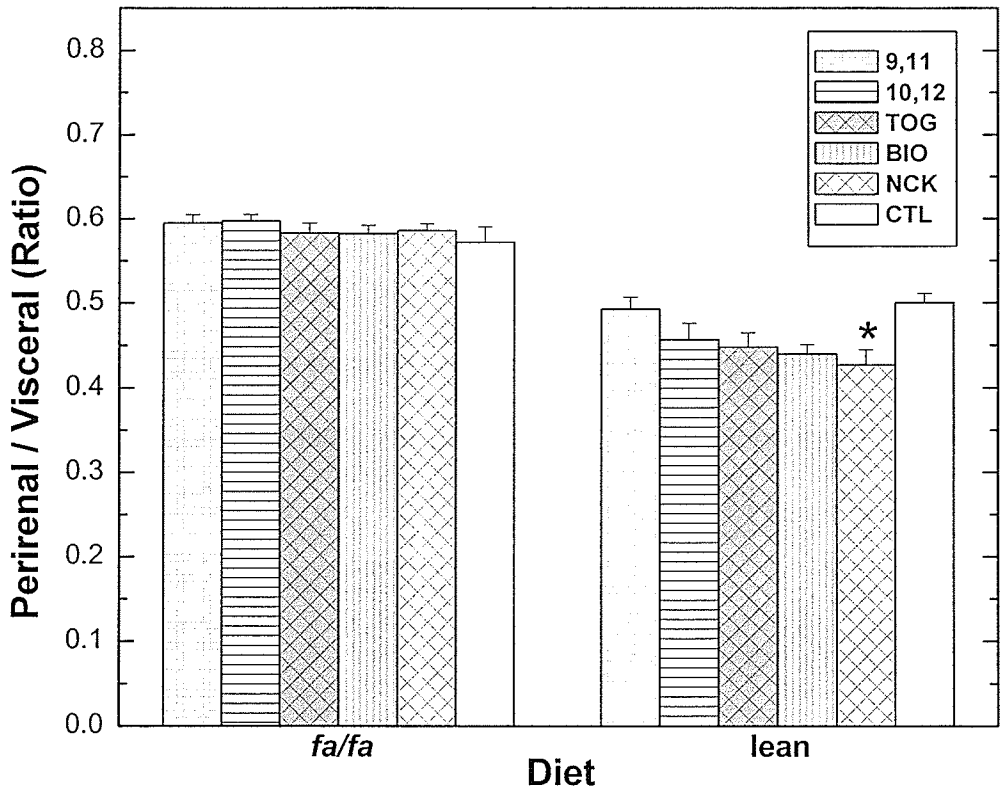


Figure 14 - The effect of 9,11, 10,12, TOG, BIO, NCK and CTL diets on perirenal / visceral fat pad ratio in Zucker rats. There was a significant diet x genotype interaction (P=0.0229). The * denotes a significant pre-determined contrast (P<0.05) between a treatment group and CTL. Data are expressed as mean ± SEM (n=10 per group).

Serum Insulin and C-Peptide

The *fa/fa* Zucker rats had an 11.7-fold higher fasting serum insulin concentrations compared to the lean Zucker rats (2813 ± 177 versus 241 ± 13 pmol/L, respectively). There was a significant diet x genotype interaction, such that the *fa/fa* rats fed the NCK had 52% lower serum insulin compared to CTL (Figure 15). There was a trend ($P>0.05$, considered as a result of Duncan's Multiple Range test) for fasting serum insulin concentrations to be lower in *fa/fa* rats fed 10,12, TOG, BIO, and NCK diets compared to 9,11 and in 10,12, TOG, and BIO diets compared to CTL.

The *fa/fa* Zucker rats had a 6.5-fold higher fasting serum C-peptide concentration compared to lean Zucker rats (8287 ± 391 versus 1099 ± 60 pmol/L, respectively; Figure 16). There was a trend ($P>0.05$, considered as a result of Duncan's Multiple Range test) for fasting serum C-peptide concentrations to be lower in *fa/fa* rats fed 10,12, TOG, BIO, and NCK diets compared to 9,11 and CTL.

Oral Glucose Tolerance Test (OGTT)

The serum glucose concentrations during the oral glucose tolerance test were assessed by a repeated measures ANOVA procedure. There was a significant diet x genotype x time interaction. However, the lean rats showed no

difference by diet group in serum glucose concentrations at any time point, and were lower than all *fa/fa* groups at 15 minutes and 60 minutes. At time 0 minutes, the *fa/fa* rats fed the 10,12 diet had a higher fasting serum glucose concentration than the other *fa/fa* rats except for BIO (Figure 17). At 15 minutes, the *fa/fa* rats fed 9,11, TOG, BIO and NCK had 14%-18% lower serum glucose concentrations compared to CTL, while the 10,12 group was not different from CTL or the other groups. At 30 minutes, the *fa/fa* rats fed the 10,12, TOG, BIO and NCK had 16%-33% lower serum glucose concentrations compared to 9,11 and CTL, while the 9,11 group was not different from CTL. At 60 minutes, the *fa/fa* rats fed the 10,12, TOG, BIO and NCK had 21%-30% lower serum glucose concentrations compared to 9,11, while the 9,11 group had 12% lower serum glucose concentrations compared to CTL.

Area Under the Curve for Glucose (AUCg)

AUCg is calculated as the area under the curve for the serum glucose results during the oral glucose tolerance test, and it is a measure of the overall serum glucose response for the full duration of the oral glucose tolerance test. The *fa/fa* Zucker rats had a 1.4-fold larger AUCg compared to the lean Zucker rats (868 ± 22 versus 607 ± 10 mmol min^{-1} L^{-1}, respectively). There was a significant diet x genotype interaction, such that the *fa/fa* rats fed the TOG, BIO and NCK had 26%, 24% and 29%, respectively, smaller AUCg compared to CTL (Figure 18). There was a trend ($P>0.05$, considered as a result of Duncan's

Multiple Range test) for AUCg to be smaller in *fa/fa* rats fed TOG, BIO, and NCK diets compared to 9,11 and in the 10,12 diet compared to 9,11 and CTL.

Homeostasis Model Assessment Index of Insulin Resistance (HOMA-IR)

Insulin resistance was estimated by calculating the HOMA-IR index [fasting serum insulin (uU/ml) x fasting plasma glucose (mmol/L)/22.5] (Katsuki A *et al.*, 2001). The *fa/fa* Zucker rats had a 14-fold higher estimated insulin resistance compared to the lean Zucker rats (165 ± 17 versus 11.6 ± 0.7 units, respectively; Figure 19). There was a significant CLA diet effect such that Zucker rats fed the 10,12, TOG, BIO and NCK had 34%, 46%, 37% and 50%, respectively, lower estimated insulin resistance compared to 9,11 regardless of genotype (Figure 20). Rats fed the TOG and NCK diets had 44% and 48%, respectively, lower estimated insulin resistance compared to CTL regardless of genotype. There was a trend ($P>0.05$, considered as a result of Duncan's Multiple Range test) for estimated insulin resistance to be lower in *fa/fa* rats fed 10,12, TOG, BIO, and NCK diets compared to 9,11 and CTL.

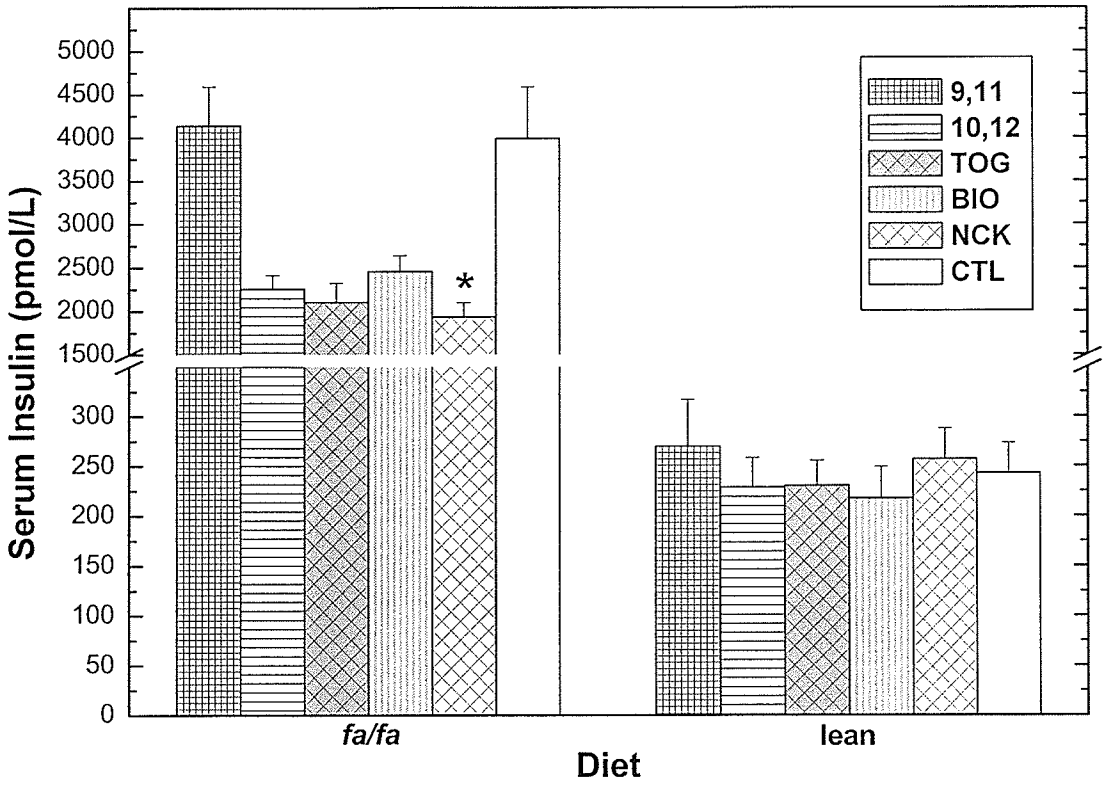


Figure 15 - The effect of 9,11, 10,12, TOG, BIO, NCK and CTL diets on fasting serum insulin concentrations in Zucker rats. There was a significant diet x genotype interaction (P=0.0108). The * denotes a significant pre-determined contrast (P<0.05) between a treatment group and CTL. Data are expressed as mean ± SEM (n=10 per group).

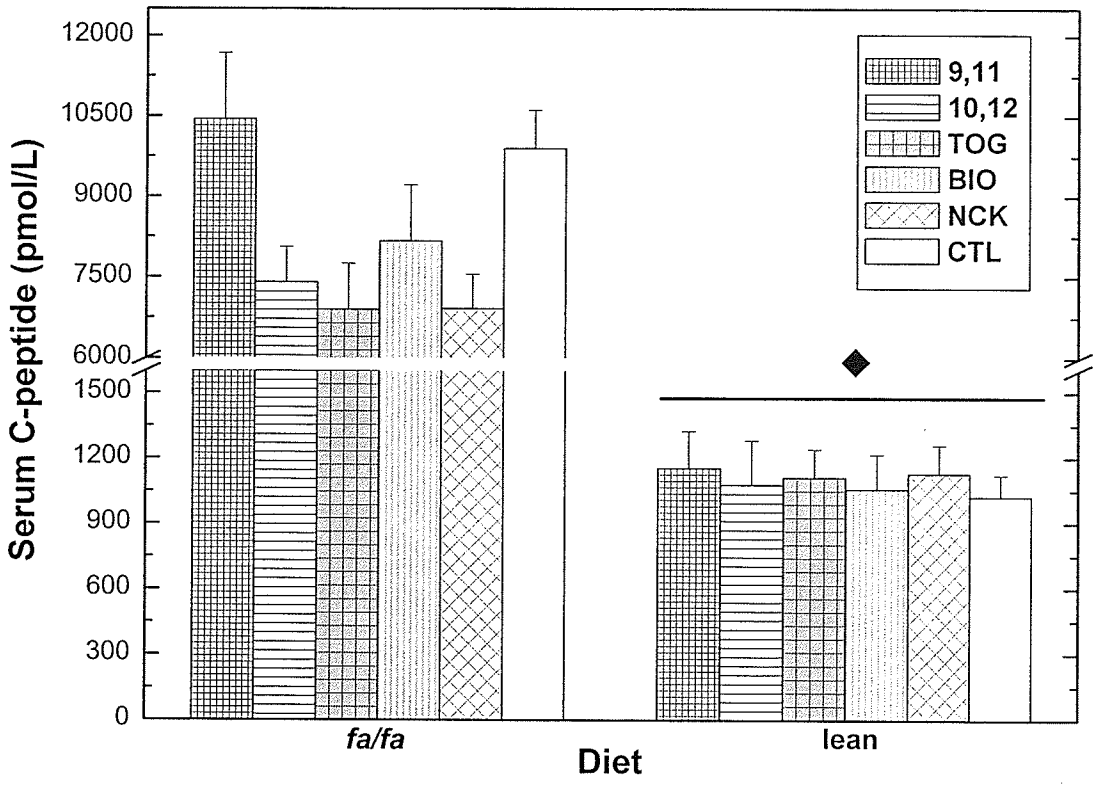


Figure 16 - The effect of 9,11, 10,12, TOG, BIO, NCK and CTL diets on fasting serum C-peptide concentrations in Zucker rats. The ◆ symbol denotes a significant genotype effect (P<0.0001). Data are expressed as mean ± SEM (n=10 per group).

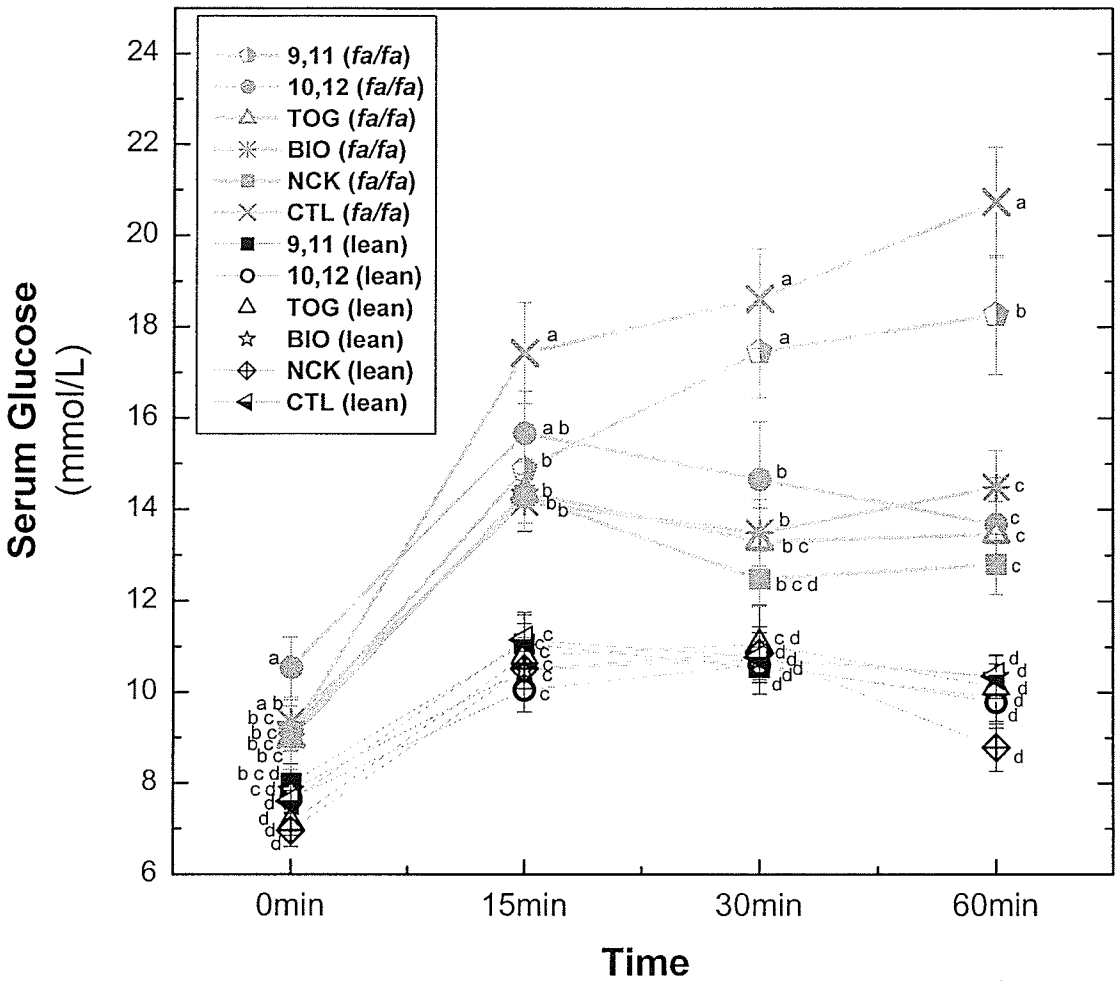


Figure 17 - The effect of 9,11, 10,12, TOG, BIO, NCK and CTL diets on serum glucose concentrations during oral glucose tolerance testing (OGTT) in Zucker rats. There was a significant diet x genotype x time interaction ($P<0.0001$). Means with different letters are significantly different ($P<0.05$), while means with the same letter are not different ($P>0.05$) by Duncan's Multiple Range test. Data are expressed as mean ± SEM (n=10 per group).

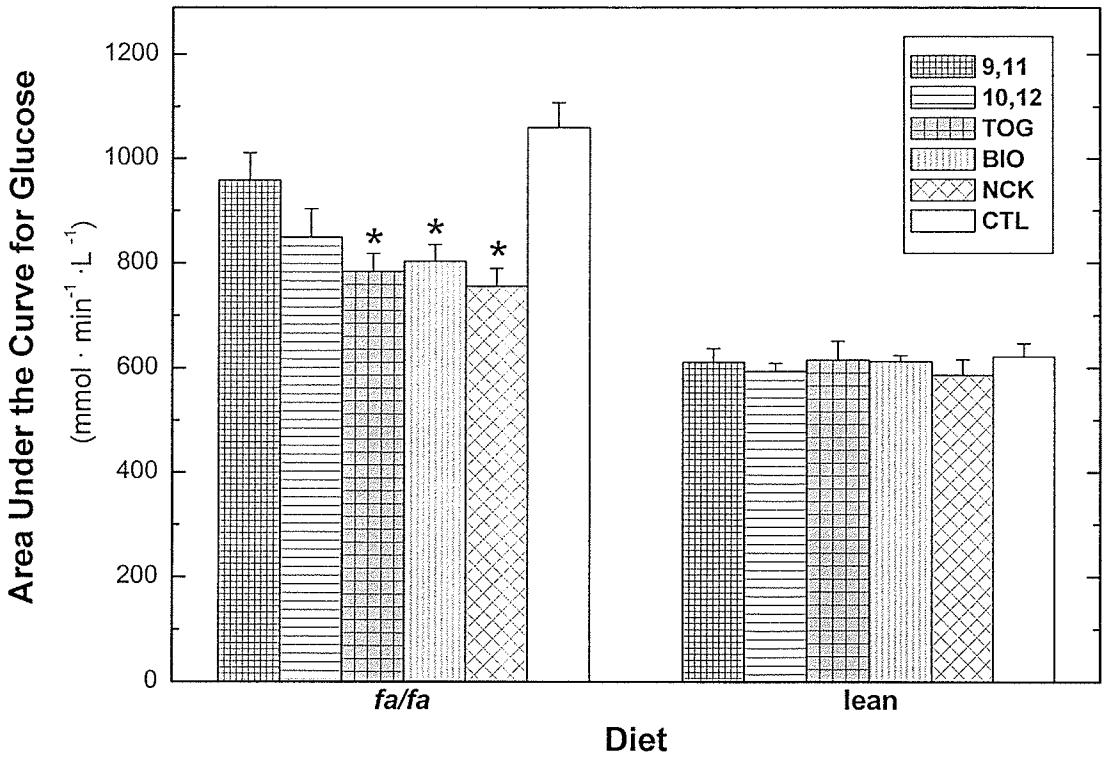


Figure 18 - The effect of 9,11, 10,12, TOG, BIO, NCK and CTL diets on area under the curve for glucose (AUCg) in Zucker rats. There was a significant diet x genotype interaction (P=0.0073). The * denotes a significant pre-determined contrast (P<0.05) between a treatment group and CTL. Data are expressed as mean ± SEM (n=10 per group).

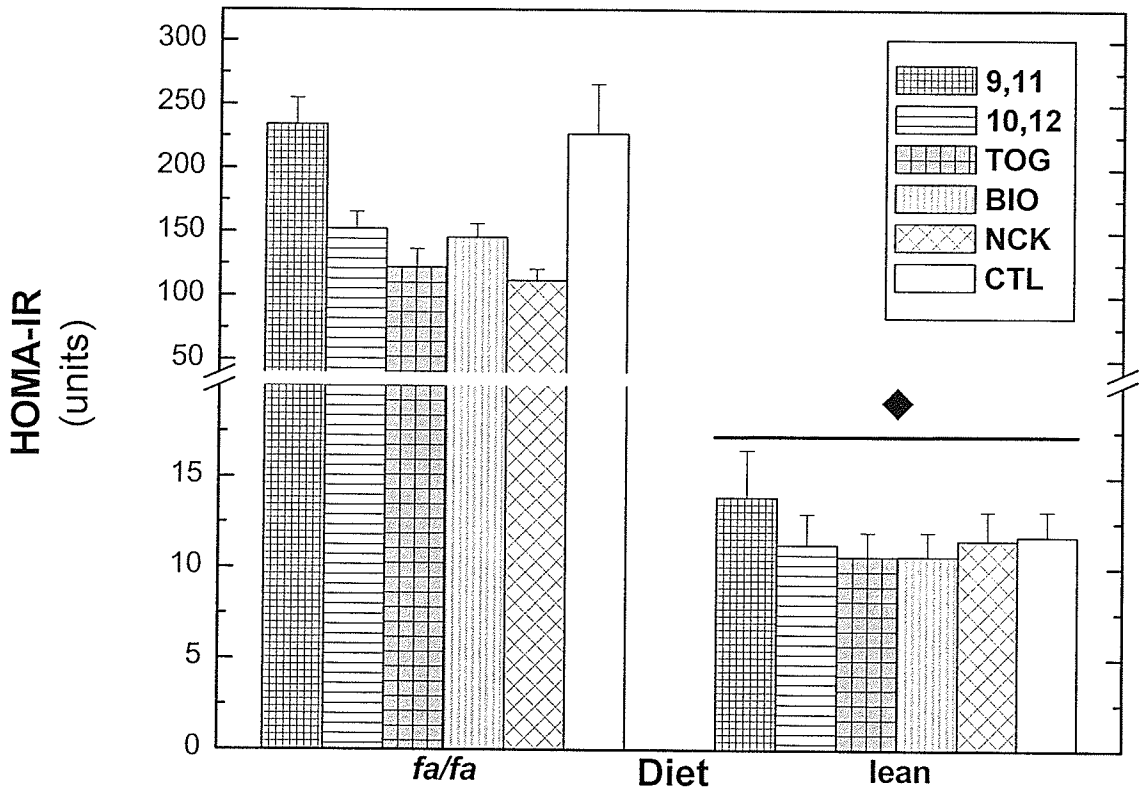


Figure 19 - The effect of 9,11, 10,12, TOG, BIO, NCK and CTL diets on homeostasis model assessment index of insulin resistance (HOMA-IR) in Zucker rats. The ◆ symbol denotes a significant genotype effect (P<0.0001). Data are expressed as mean ± SEM (n=10 per group).

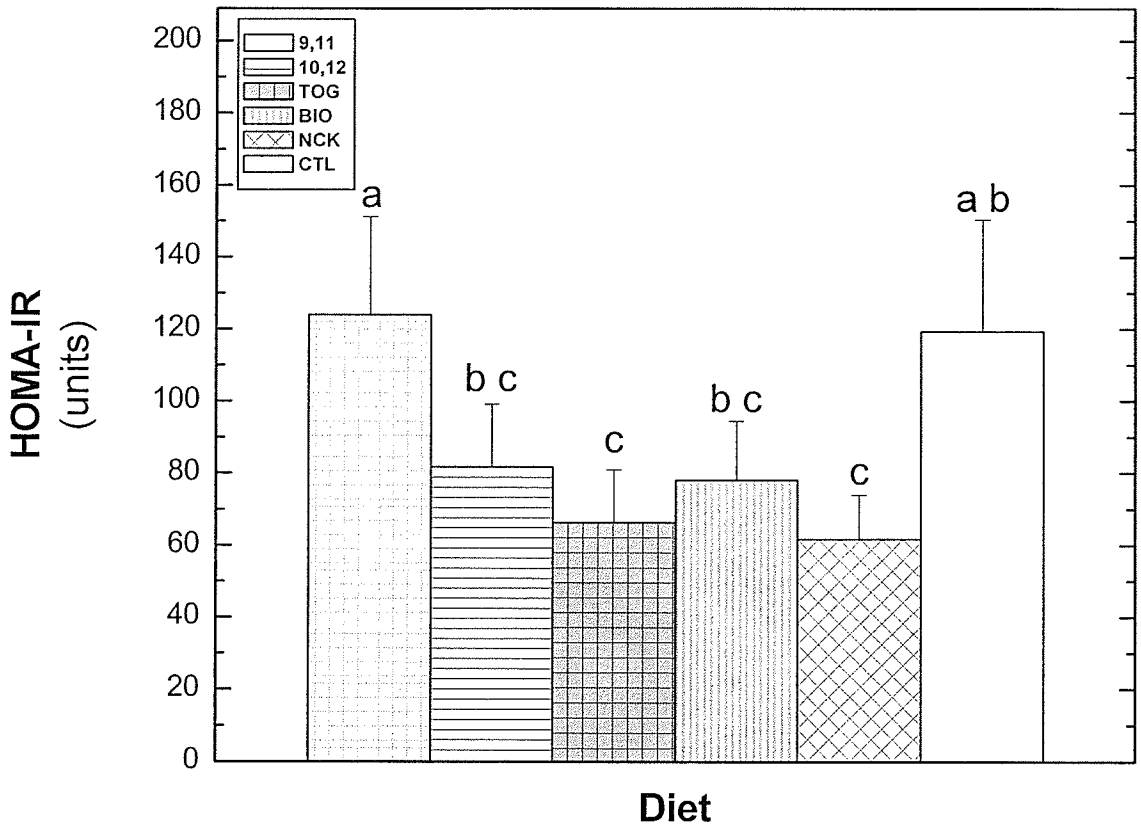


Figure 20 - The effect of 9,11, 10,12, TOG, BIO, NCK and CTL diets on homeostasis model assessment index of insulin resistance (HOMA-IR) in Zucker rats. There was a significant CLA diet effect (P=0.0009). Means with different letters are significantly different from one another (P<0.05), while means with the same letter are not different from one another (P>0.05) by Duncan's Multiple Range test. Data are expressed as mean ± SEM (n=20 per group).

Serum Triglycerides and Free Fatty Acids

The *fa/fa* Zucker rats had a 7.3-fold higher serum triglyceride concentrations compared to the lean Zucker rats (4.88 ± 0.30 versus 0.58 ± 0.03 mmol/L, respectively; Figure 21), but there was no effect of dietary CLA treatment.

The *fa/fa* Zucker rats had 2.5-fold greater serum free fatty acid concentrations compared to the lean Zucker rats (1.27 ± 0.04 versus 0.52 ± 0.02 mmol/L, respectively; Figure 22), but there was no effect of dietary CLA treatment.

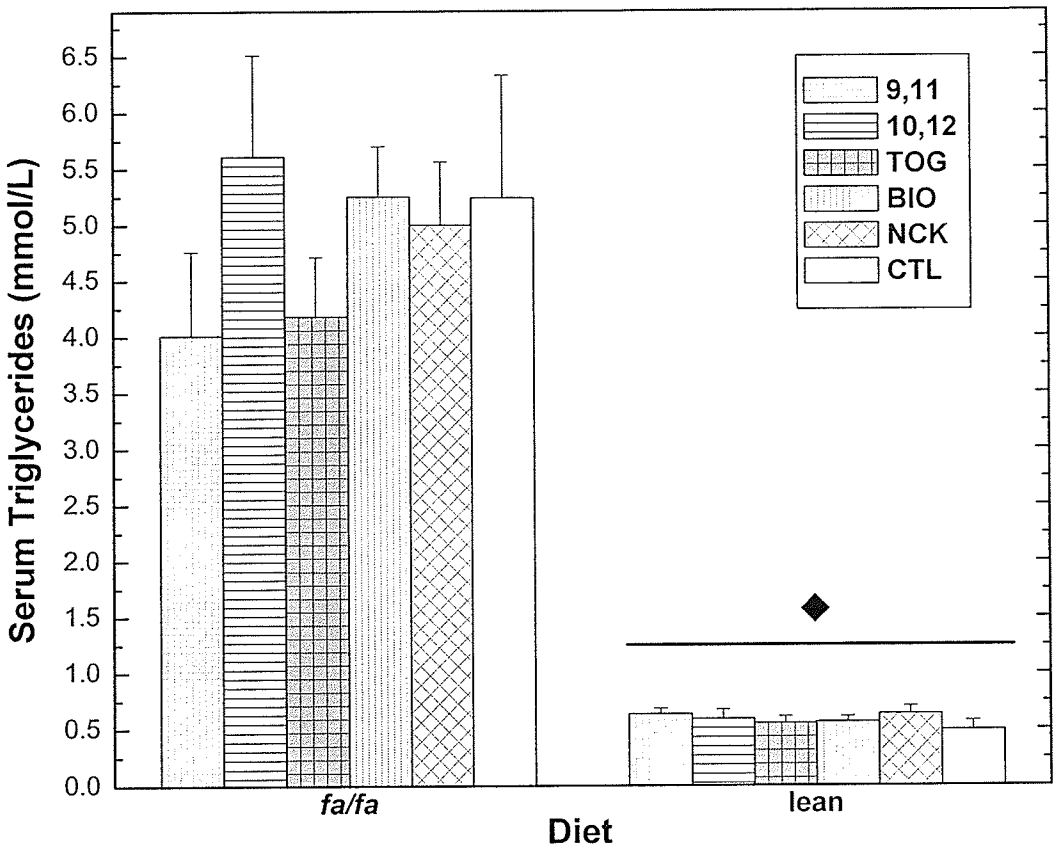


Figure 21 - The effect of 9,11, 10,12, TOG, BIO, NCK and CTL diets on serum triglyceride levels in Zucker rats. The ◆ symbol denotes a significant genotype effect (P<0.0001). Data are expressed as mean ± SEM (n=10 per group).

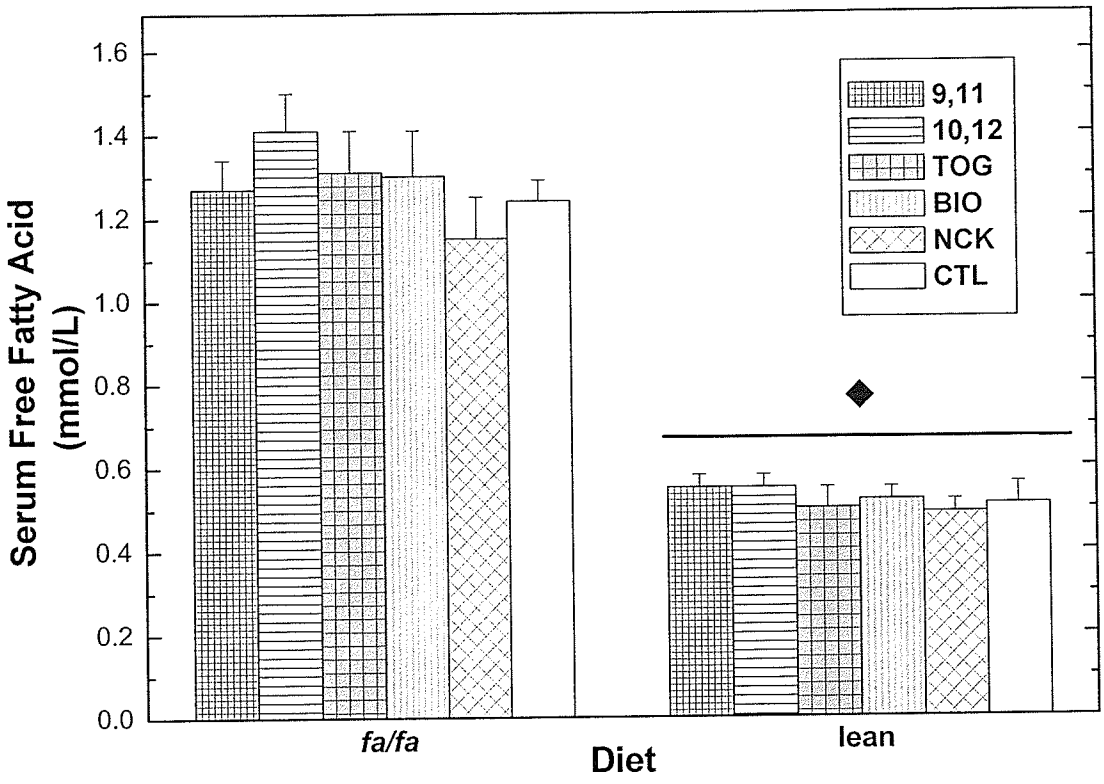


Figure 22 - The effect of 9,11, 10,12, TOG, BIO, NCK and CTL diets on serum free fatty acid levels in Zucker rats. The ◆ symbol denotes a significant genotype effect (P<0.0001). Data are expressed as mean ± SEM (n=10 per group).

Skeletal Muscle AMPK-α and phospho-AMPK-α

There was no significant main effect of genotype or dietary CLA on skeletal muscle AMPK-α or phospho-AMPK-α levels (Figures 23 & 24), or the ratio of skeletal muscle phospho-AMPK-α / AMPK-α (Figure 25).

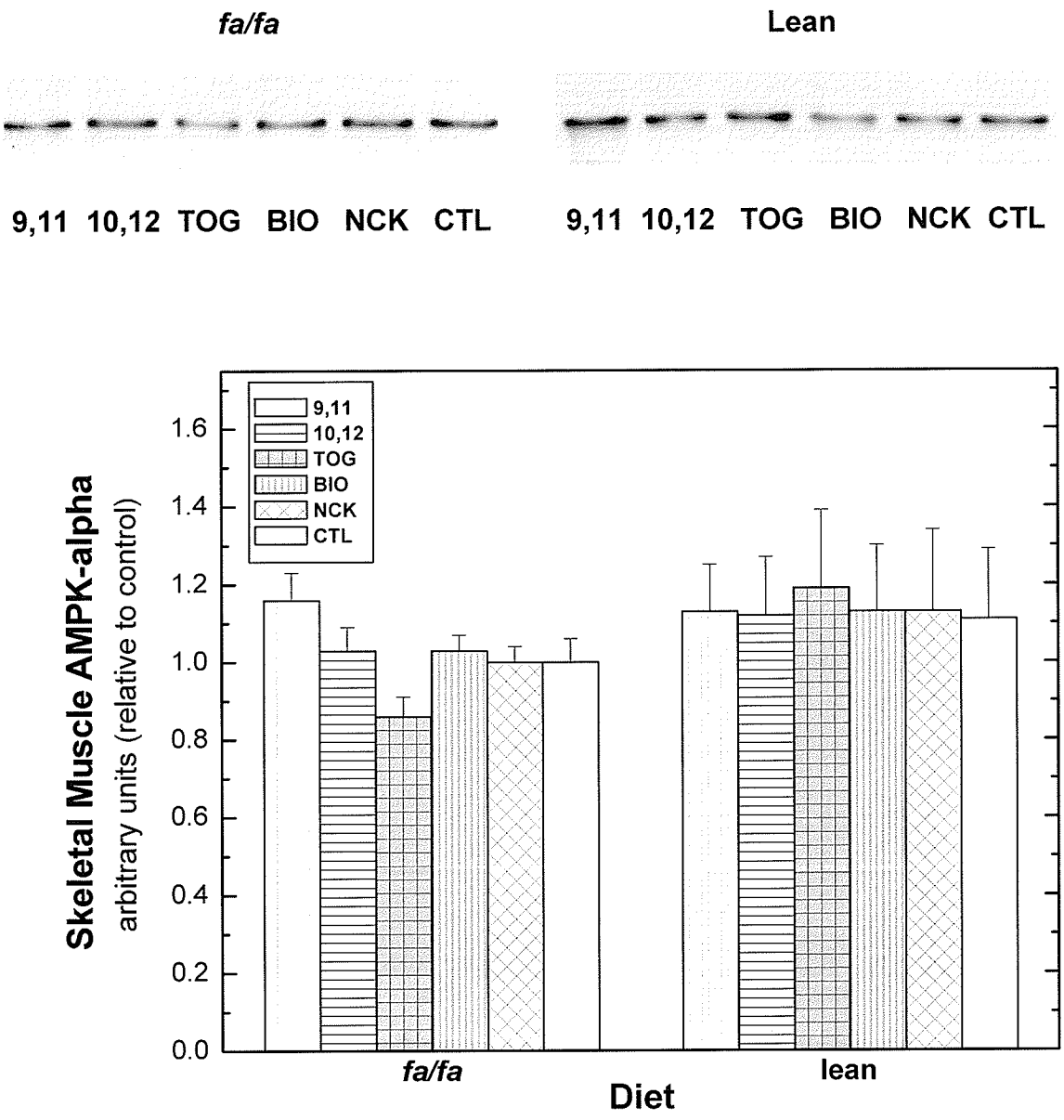
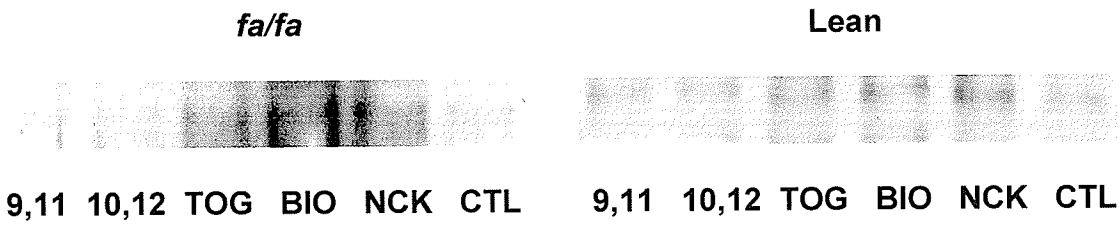


Figure 23 - The effect of 9,11, 10,12, TOG, BIO, NCK and CTL diets on skeletal muscle AMPK-α levels in Zucker rats. Data are expressed as mean ± SEM (n=7 per group). There were no significant main effects.



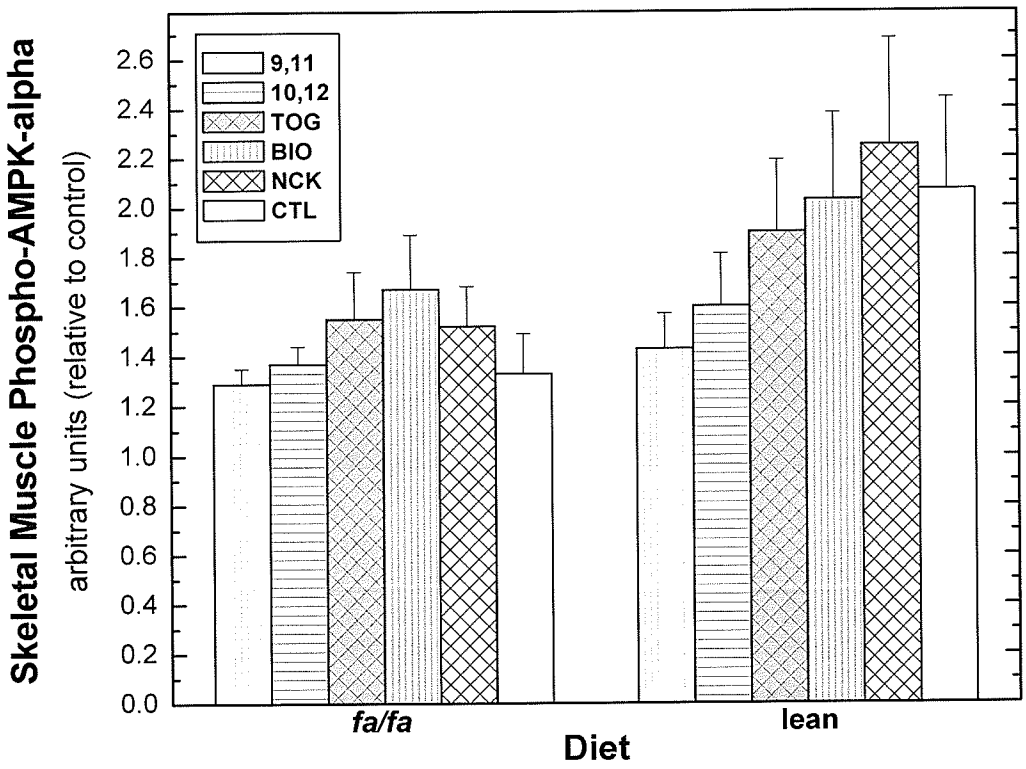


Figure 24 - The effect of 9,11, 10,12, TOG, BIO, NCK and CTL diets on skeletal muscle phospho-AMPK-α levels in Zucker rats. Data are expressed as mean ± SEM (n=6-7 per group). There were no significant main effects.

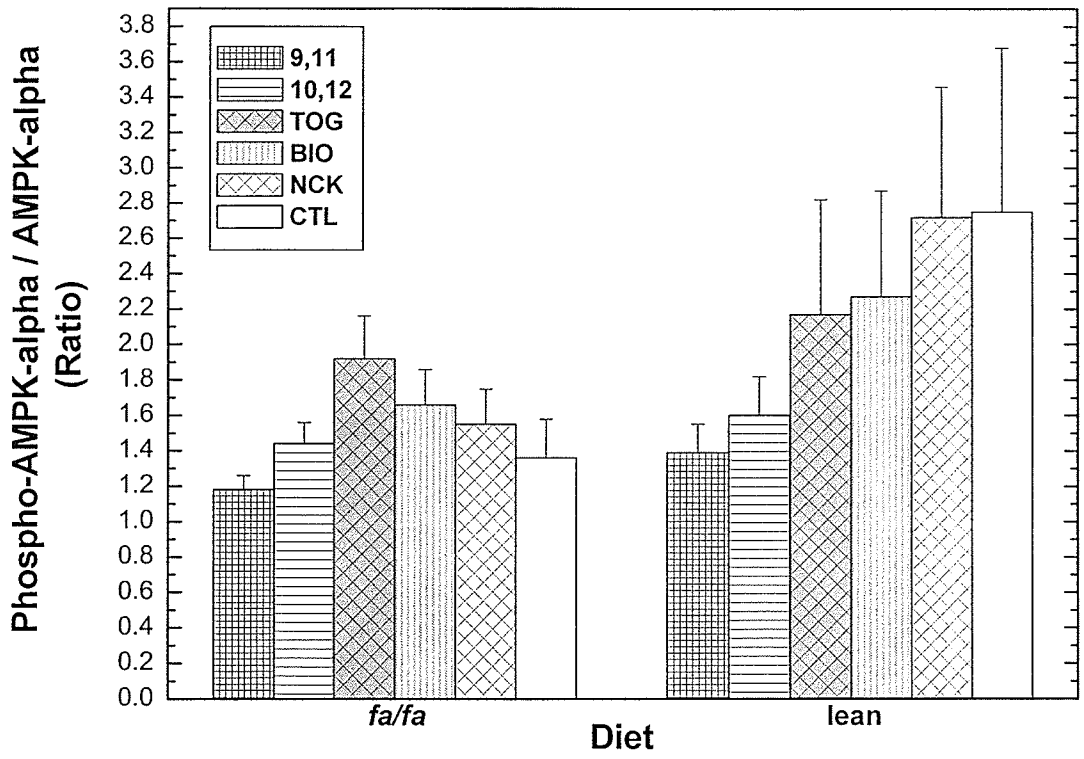


Figure 25 - The effect of 9,11, 10,12, TOG, BIO, NCK and CTL diets on skeletal muscle phospho-AMPK-α / AMPK-α ratios in Zucker rats. Data are expressed as mean ± SEM (n=6 per group). There were no significant main effects.

Skeletal Muscle TNF-α

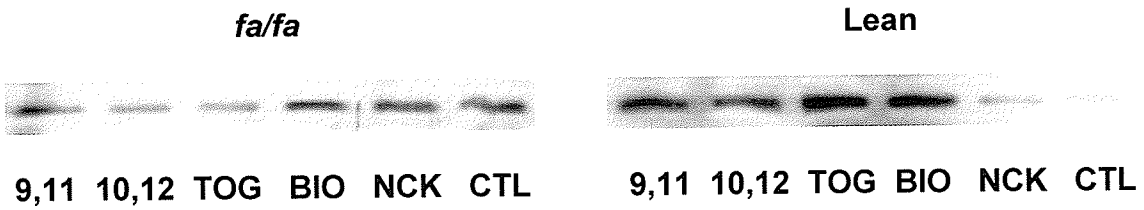
TNF-α has two forms and is expressed both as a membrane bound (26 kDa) protein and a physiologically active (19 kDa) form (Pallandino *et al.*, 2003). The *fa/fa* Zucker rats had a 1.4-fold lower skeletal muscle TNF-α (19 kDa) level compared to the lean Zucker rats (1.08 ± 0.08 versus 1.48 ± 0.18, respectively; Figure 26), but there was no effect of dietary CLA treatment. There was a trend ($P>0.05$, considered as a result of Duncan's Multiple Range test) for skeletal muscle TNF-α (19 kDa) levels to be higher in lean rats fed the TOG diet compared to CTL.

The *fa/fa* Zucker rats had a 1.8-fold lower skeletal muscle TNF-α (26 kDa) level compared to the lean Zucker rats (0.85 ± 0.10 versus 1.51 ± 0.19, respectively; Figure 27), but there was no effect of dietary CLA treatment. There was a trend ($P>0.05$, considered as a result of Duncan's Multiple Range test) for skeletal muscle TNF-α (26 kDa) levels to be higher in lean rats fed TOG diet compared to CTL.

The *fa/fa* Zucker rats had a 1.6-fold lower total skeletal muscle TNF-α level (19 kDa + 26 kDa) compared to the lean Zucker rats (1.93 ± 0.17 versus 2.99 ± 0.35, respectively; Figure 28), but there was no effect of dietary CLA treatment. There was a trend ($P>0.05$, considered as a result of Duncan's

Multiple Range test) for total skeletal muscle TNF-α (19 kDa + 26 kDa) levels to be higher in lean rats fed TOG diet compared to CTL.

The *fa/fa* Zucker rats had a 1.1-fold higher skeletal muscle TNF-α ratio (19 kDa/(19 kDa + 26 kDa)) compared to the lean Zucker rats (0.57 ± 0.02 versus 0.50 ± 0.02, respectively; Figure 29), but there was no effect of dietary CLA treatment.



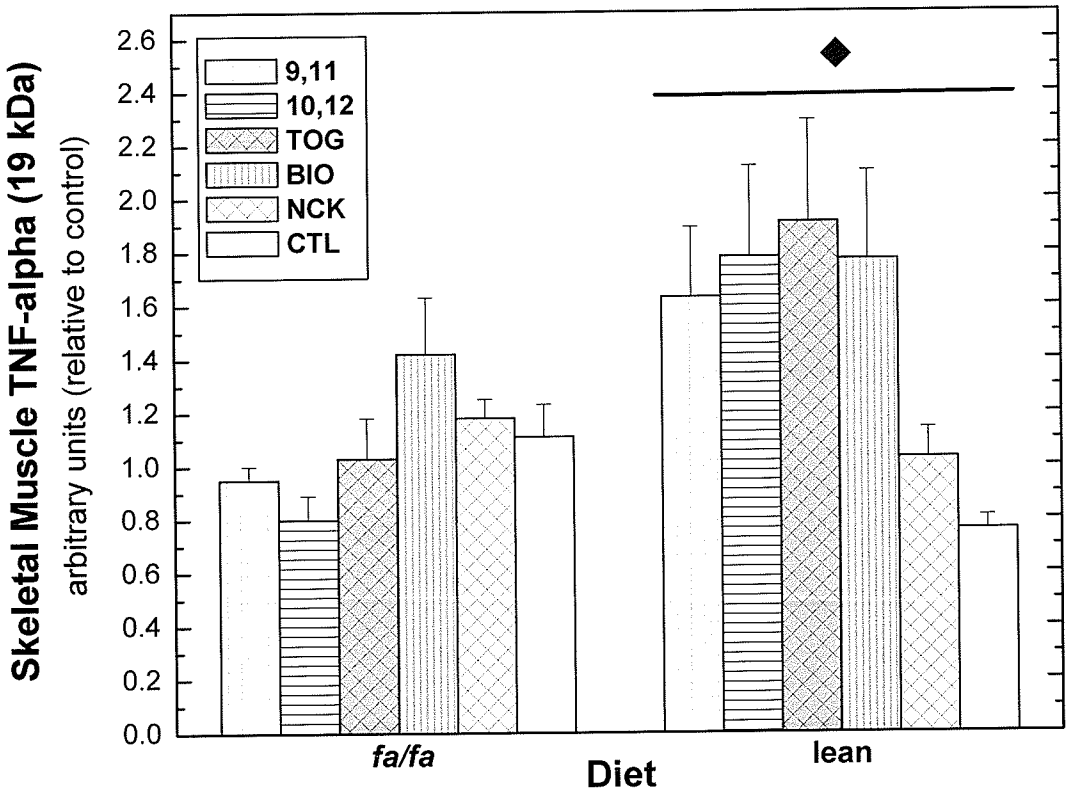
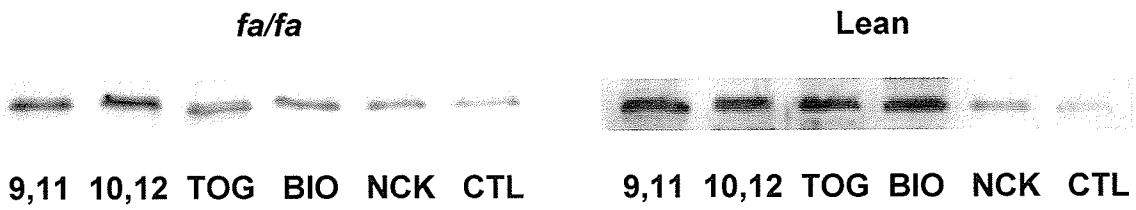


Figure 26 - The effect of 9,11, 10,12, TOG, BIO, NCK and CTL diets on skeletal muscle TNF-α (19 kDa) levels in Zucker rats. The ◆ symbol denotes a significant genotype effect (P<0.0479). Data are expressed as mean ± SEM (n=4 per group).



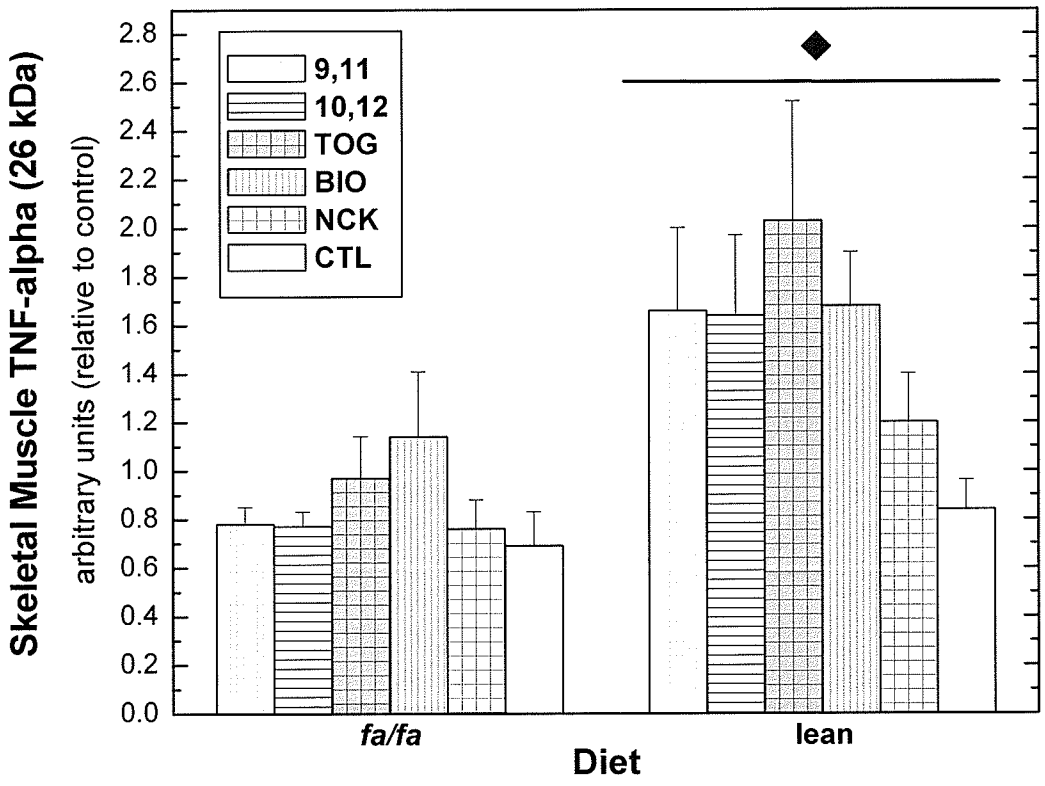


Figure 27 - The effect of 9,11, 10,12, TOG, BIO, NCK and CTL diets on skeletal muscle TNF-α (26 kDa) levels in Zucker rats. The ◆ symbol denotes a significant genotype effect (P=0.0078). Data are expressed as mean ± SEM (n=4 per group).

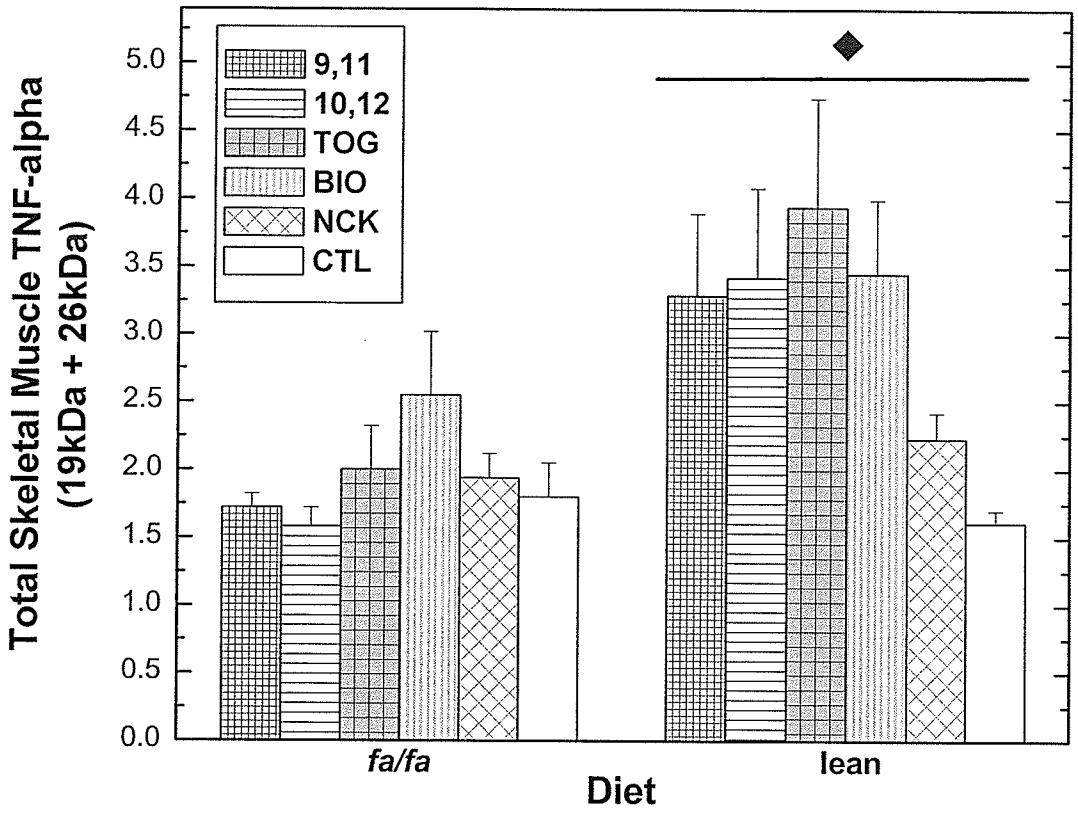


Figure 28 - The effect of 9,11, 10,12, TOG, BIO, NCK and CTL diets on total skeletal muscle TNF-α levels in Zucker rats. The ◆ symbol denotes a significant genotype effect (P=0.0105). Data are expressed as mean ± SEM (n=4 per group).

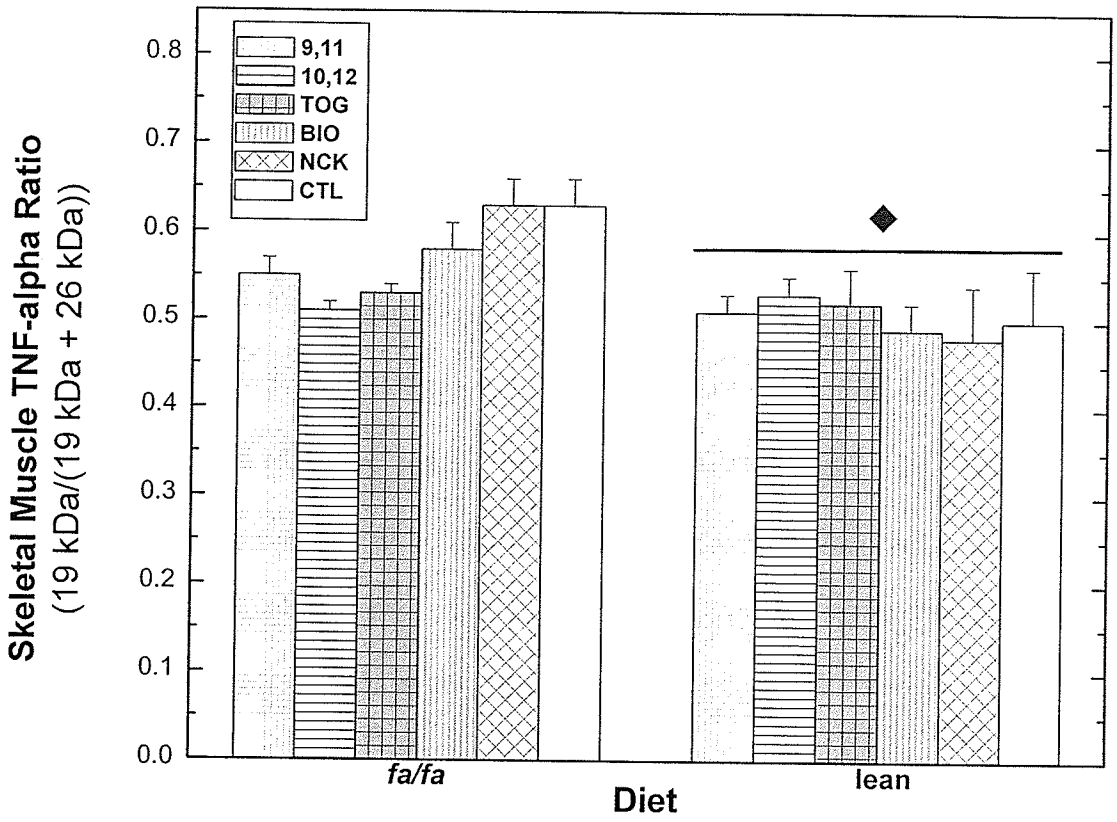


Figure 29 - The effect of 9,11, 10,12, TOG, BIO, NCK and CTL diets on skeletal muscle TNF-α ratios in Zucker rats. The ◆ symbol denotes a significant genotype effect (P=0.0339). Data are expressed as mean ± SEM (n=4 per group).

5. DISCUSSION

Body Weight and Feed Intake

At the end of our 8 week study, the *fa/fa* Zucker rats were 14 weeks old and had a 1.7-fold higher final body weight compared to the lean Zucker rats (Figure 1). Similarly, Sisk *et al.* (2001) reported a 1.4-fold higher final body weight in 12-13 week old *fa/fa* vs. lean Zucker rats at the end of a 5 week study. The *fa/fa* Zucker rats are obese and have a higher body weight than the lean Zucker rats because they carry an autosomal recessive *fa* gene for obesity, which leads to insulin resistance, hyperinsulinemia and glucose intolerance (White & Martin, 1997). The *fa* mutation is a point mutation that results in an alteration of one amino acid, causing decreased functional effectiveness of the leptin receptor. It is thought that this deficiency in the leptin receptor pathway prevents critical hypothalamic areas from receiving significant cues on energy status. Therefore, if the hypothalamus does not receive, sense, or properly integrate the nutritional state of the animal, as in the *fa/fa* Zucker rat, a perceived negative energy balance and subsequent hyperphagia occurs, resulting in obesity (White & Martin, 1997).

Not surprisingly, the *fa/fa* Zucker rats had a 1.6-fold higher total feed intake and feed intake per day compared to the lean Zucker rats, due to

hyperphagia (Figures 2 & 3). Similarly, Sisk *et al.* (2001) reported a 1.3-fold higher feed intake per day of *fa/fa* rats compared to lean rats.

However, there was no effect of dietary CLA treatment on final body weight (Figure 1). Previous studies originally done with mice have shown that CLA reduces final body weights when compared to control-fed mice, leading us to believe that CLA would have beneficial effects on reducing overall body weight. One such study showed that male AKR/J mice fed a 1% CLA mixture for durations of 4, 6, 8, or 12 weeks had significantly lower final body weights at each time point compared to control mice (Delany *et al.*, 1999). Conversely, studies done in other species have not shown CLA to be beneficial in lowering overall final body weight. Our results in *fa/fa* Zucker rats are in agreement with Sisk *et al.* (2001) who showed that administration of a 0.5% CLA mixture for 5 weeks had no effect on final body weights in either female or male lean and *fa/fa* Zucker rats. Nagao *et al.* (2003) found that the administration of a 1.0% CLA mixture for 8 weeks had no effect on final body weights in male *fa/fa* rats. In healthy adult women, consumption of 3.0 g CLA mixture/day for 12 weeks did not benefit in terms of final body weight (Zambell *et al.*, 2000). In contrast, Ryder *et al.* (2001) found that ZDF rats fed 1.5% CLA consisting of primarily the c9,t11 CLA isomer had a higher final body weight compared to the ZDF rats fed 1.5% CLA consisting of 50:50 mixture of c9,t11 CLA and t10,c12 CLA. However, this study was only conducted for 14 days, making it difficult to make a comparison

with longer term studies. As well the ZDF rat is a different strain of rat, which exhibits overt Type 2 diabetes.

Despite dietary CLA treatment having no effect on final body weights, the *fa/fa* rats fed the 10,12 diet had 16% lower total feed intake and feed intake per day compared to CTL-fed *fa/fa* rats (Figures 2 & 3). Similarly, Clement *et al.* (2002) observed that female C57BL/6J mice fed 0.4% t10,c12 CLA had 29% lower feed intake per day. This suggests that the single isomer t10,c12 CLA is responsible for reduced feed intake in the obese *fa/fa* Zucker rat model. It is possible that the t10,c12 CLA isomer may have positively acted upon the deficient leptin receptors reducing hyperphagia, however, this is inconclusive. Interestingly, despite a reduced total feed intake and feed intake per day, the *fa/fa* rats fed the 10,12 diet had no decrease in final body weight (Figure 1). This result implies that obese *fa/fa* Zucker rats required less 10,12 diet consumption to achieve a similar final body weight compared to the rest of the diet groups. This demonstrates that the t10,c12 CLA isomer is linked to weight gain in conjunction with a lower feed intake. It is possible that the t10,c12 CLA isomer lowers thermogenesis by having a suppressive effect on heat production. It is also possible that changes in certain tissue(s) such as the slight increase in epididymal fat pad weight (Figure 6), or perhaps lean mass are responsible for the similar final body weights despite decreased feed consumption. At this point, however, no conclusion can be reached.

Feed Efficiency Ratio

The *fa/fa* Zucker rats had a 1.2-fold higher FER compared to the lean Zucker rats. The *fa/fa* Zucker rats have a higher FER compared to the lean rats due to a greater change in body weight over the course of the 8 week study in comparison to their total feed intake. This implies that the thermogenesis of the *fa/fa* Zucker rats is suppressed compared to the lean Zucker rats, leading to a reduced metabolism and a subsequent increase in adipose tissue. Similarly, Sisk *et al.* (2001) reported a 1.2-fold higher FER of *fa/fa* rats compared to lean rats. Considering that the *fa/fa* rats are an obese model, increased FER is not a desirable trait, unless lean body mass was being enhanced.

The *fa/fa* rats fed the TOG diet had 13% higher FER and the 10,12 diet showed a trend for increased FER compared to CTL (Figure 4). This suggests that the *fa/fa* rats fed the t10,c12 CLA isomer alone required less total feed intake to achieve the same gain in body weight as CTL rats over the 8 week study, and this effect was more pronounced when t10,c12 CLA was provided as a 50:50 mixture with c9,t11 CLA. Similarly, Ostrowska *et al.* (1999) showed that Female cross-bred (Large White x Landrace) pigs fed a CLA mixture [c11,t13 (18%), c10,t12 (30%), c9,t11 (25%) and c8,t10 (14%), along with traces of seven other isomers] had a 6.3% higher FER compared to control. However, the species as well as the CLA isomer constituents in the Ostrowska *et al.*, (1999) diet formulation differed form our TOG diet, that combined CLA as a 0.8% 50:50

mixture of c9,t11 CLA and t10,c12 CLA. In contrast, Sisk *et al.* (2001) showed no differences in FER of *fa/fa* rats administered a CLA mixture diet. Possible reasons for this discrepancy may be that the Sisk *et al.* (2001) study lasted for only 5 weeks compared to our 8 week study. Another reason may be differences in diet formulation and composition and amount of CLA. They used a 0.5% two isomer CLA mixture with varying amounts of other CLA isomers, while our 10,12 diet contained 0.4% t10,c12 CLA and our TOG diet combined 0.4% c9,t11 CLA and 0.4% t10,c12 CLA.

Adipose Tissue Mass: Epididymal, Perirenal and Visceral (Absolute (g) and Relative to Body Weight (g/100g))

The *fa/fa* Zucker rats had a 3.6-fold higher epididymal fat pad weight and 2.1-fold higher epididymal fat pad weight relative to body weight compared to the lean Zucker rats (Figures 5 & 10). Similarly, Sisk *et al.* (2001) reported a 3.9-fold higher epididymal fat pad weight in *fa/fa* vs. lean Zucker rats at the conclusion of their 5 week study.

Furthermore, there was a 5.8-fold higher perirenal fat pad weight and a 3.5-fold higher perirenal fat pad weight relative to body weight in the *fa/fa* Zucker rats compared to the lean Zucker rats (Figures 7 & 11).

Visceral fat pad weight, more commonly known as the internal adipose tissue, was obtained by adding epididymal and perirenal fat pad weights together. This resulted in a 4.6-fold higher visceral fat pad weight and a 2.8-fold higher visceral fat pad weight relative to body weight in the *fa/fa* Zucker rats compared to the lean Zucker rats (Figures 8 & 12). Sisk *et al.* (2001) showed similar findings of 5.8-fold higher total fat pad weights and a 4.3-fold higher total fat pad weights relative to body weight in the *fa/fa* Zucker rats compared to the lean Zucker rats. This slight dissimilarity between our study and Sisk *et al.* (2001) can probably be explained by inter-animal variability, which is impossible to control for, and may be responsible for the slightly higher differences observed between *fa/fa* and lean Zucker rats. As well, the method Sisk *et al.* (2001) used to calculate their total fat pad weight or visceral fat differed from our method, as they included the inguinal, retroperitoneal and epididymal fat pads in their calculation.

There was a significant CLA diet effect such that Zucker rats fed 10,12, TOG and BIO diets had higher (11-24%) epididymal fat pad weights compared to 9,11 and CTL regardless of genotype (Figure 6). There was a trend (Figure 5 & 10) for absolute epididymal fat pad weights and epididymal fat relative to body weight to be higher in *fa/fa* rats fed 10,12, TOG, BIO, and NCK diets compared to 9,11 and CTL. However, Sisk *et al.* (2001) reported no effect of a dietary CLA mixture on epididymal fat pad weights in Zucker rats, possibly because their study only lasted 5 weeks and their CLA mixture differed from ours. Our findings

show that the t10,c12 CLA isomer individually and more pronounced in combination with other CLA isomers (purified 50:50 mixture with c9,t11 CLA and/or a two or four isomer CLA mixture) resulted in higher accumulations of epididymal fat pad weight (absolute amount and relative to body weight), while the c9,t11 CLA isomer had no effect. Ryder *et al.* (2001) showed that ZDF rats fed 1.5% 50:50 CLA mixture had 12% lower epididymal fat pad weights compared to the 1.5% c9,t11 CLA treatment group and 17% lower epididymal fat pad weights compared to the ZDF control group. This contradicts our findings, since our *fa/fa* 9,11 group had the lowest epididymal fat pad weights, while our *fa/fa* TOG diet, a combined CLA as a 50:50 mixture of c9,t11 CLA and t10,c12 CLA had the highest. This difference might be explained by their short study duration of 14 days and the fact that both of their CLA diets consisted of 1.5% concentration, while ours were 0.4% for the 9,11 diet (c9,t11 CLA isomer) and 0.8% for the TOG diet, a combined CLA 50:50 mixture of c9,t11 CLA and t10,c12 CLA, with lipid contributing 8.5% (wt/wt) of the diet despite CLA amounts.

There was no effect of dietary CLA treatment on perirenal fat pad weights, however, there was a trend (Figure 7) for perirenal fat pad weights to be higher in *fa/fa* rats fed 10,12, TOG, BIO, and NCK diets compared to 9,11 and CTL. Consequently, the *fa/fa* rats fed the TOG, BIO and NCK had higher (19-22%) perirenal fat pad weights relative to body weight compared to CTL (Figure 11). These findings agree with the trends observed for epididymal fat of the *fa/fa* rats. This again implies that the t10,c12 CLA isomer individually and more pronounced

in combination with other CLA isomers (purified 50:50 mixture with c9,t11 CLA and/or a two or four isomer CLA mixture) resulted in higher accumulations of perirenal fat pad weight (absolute amount and relative to body weight), while the c9,t11 CLA isomer alone had no effect.

There was a significant CLA diet effect such that Zucker rats fed the 10,12 and TOG diets had 13% and 18%, respectively, higher visceral fat pad weights compared to 9,11 and CTL groups regardless of genotype (Figure 9), with a concomitant trend (Figure 8) for visceral fat pad weights to be higher in *fa/fa* rats fed 10,12, TOG, BIO, and NCK diets. Consequently, the *fa/fa* rats fed the TOG, BIO and NCK diets had higher (17-22%) visceral fat pad weights relative to body weight when compared to 9,11 and CTL (Figure 12). Sisk *et al.* (2001) did not find a significant CLA diet effect such as we did, however, they did find a diet x genotype interaction, observing that feeding a CLA mixture to the *fa/fa* genotype resulted in 11% higher total visceral fat pad weights than the control diet. Conversely, Henriksen *et al.* (2003) showed that a 50:50 mixture of c9,t11 CLA and t10,c12 CLA, along with c9,t11 CLA singly had no effect on total visceral fat pad weights in the *fa/fa* Zucker rats, while the t10,c12 isomer showed a 12.5% decrease in total visceral fat pad weights compared to the control diet in the *fa/fa* Zucker rats. However, this study only lasted for 21 days compared to our 8 week study suggesting that possible dose duration may play a factor in the differing results. As well, Henriksen *et al.* (2003) studied the effects on female *fa/fa*

Zucker rats, while ours were male, possibly rendering differing results due to gender differences.

Our results demonstrate that the c9,t11 CLA isomer is responsible for lower total accumulation of internal body fat (absolute amount and relative to body weight) compared to other CLA dietary treatments, but the 9,11 group was not different compared to CTL. Conversely, the t10,c12 CLA isomer individually and more pronounced in combination with other CLA isomers (purified 50:50 mixture with c9,t11 CLA and/or a two or four isomer CLA mixture) was responsible for the highest accumulations of internal body fat (absolute amount and relative to body weight) compared to CTL. Interestingly, there was no change in total body weight (Figure 1) of *fa/fa* rats fed the c9,t11 CLA isomer, despite the lower total visceral body fat (absolute amount or relative to body weight). Consequently, these rats must have had other tissue(s) adding to the total body weight, such as hepatic steatosis observed by Noto *et al.* (2004) or possibly increased lean mass, or enhanced bone density despite lowered body fat. The 50:50 mixture of c9,t11 CLA and t10,c12 CLA showed the highest accumulation of visceral body fat (absolute amount and relative to body weight) in the *fa/fa* rats, yet these rats showed no increase in total body weight. As a result, these rats must have had changes in other tissue(s), such as reduced hepatic steatosis observed by Noto *et al.* (2004) or possibly reduced lean mass or even decreased bone density when compared to the other groups.

Overall, this indicates that the t10,c12 CLA isomer is linked to higher accumulations of adipose tissue, the effect is more pronounced in combination with other CLA isomers in obese insulin-resistant rats, and the c9,t11 CLA isomer has no effect. As a consequence, our study has yielded results that cast reservation on the universality of the benefits of CLA as far as reducing fat deposition during a relatively long-term study in comparison to other CLA studies, particularly in an obese insulin-resistant state.

Adipose Tissue Ratios: Epididymal/Visceral and Perirenal/Visceral

The *fa/fa* Zucker rats had a 1.3-fold lower epididymal/visceral fat pad ratio compared to the lean Zucker rats, while having a 1.3-fold higher perirenal/visceral fat pad ratio compared to the lean Zucker rats. Whether this means that the number of perirenal fat cells is simply increased in the *fa/fa* Zucker rat genotype or if the perirenal fat cell volume or size is increased in the *fa/fa* genotype when compared to the lean genotype is undetermined. Sisk *et al.* (2001) found that the average fat cell diameter and volume were decreased in the lean genotype but increased in the *fa/fa* genotype in response to dietary CLA, however, fat cell numbers were not affected by dietary CLA.

Despite visceral fat pad weights either being lower or higher amongst the groups within the *fa/fa* genotype, CLA had no effect on the distribution of epididymal or perirenal fat in relation to visceral fat in these groups. This means

that the total percentages of epididymal and perirenal fat pad weights were relatively similar amongst all *fa/fa* rat groups.

The lean rats fed the NCK diet [4 CLA isomer mixture and highest total amount of CLA (1.9%)] showed a higher (13%) epididymal and lower (15%) perirenal fat pad ratio relative to visceral fat compared to CTL (Figures 13 & 14). Whether this is of any importance, or was simply a result of animal variability is undetermined. The fact that the lean rats fed the NCK diet did not have significantly less total visceral fat compared to CTL demonstrates that total fat deposition was not altered, only the location of the specific fat depots. Another possibility may have been a decrease in perirenal adipocyte size or perhaps apoptosis of adipocytes, however, further examination is required.

Serum Insulin and C-Peptide

The *fa/fa* Zucker rats had an 11.7-fold higher fasting serum insulin concentration compared to the lean Zucker rats. Serum insulin levels are higher in the *fa/fa* rats compared to the lean genotype due to the mutation in the *fa* gene, which leads to a deficiency in the leptin receptors causing hyperphagia and ultimately obesity, leading to a concomitant increase in insulin resistance. It has been shown that peripheral tissues of the *fa/fa* rats are insulin resistant when tested *in vivo* (Terrettaz *et al.*, 1983). King *et al.* (1992) observed that there is a failure of translocation of glucose transporters GLUT1 and GLUT4 in the *fa/fa*

rats, decreasing the ability of insulin to stimulate glucose uptake in skeletal muscle. Furthermore, in the *fa/fa* rats, activation of A_1 adenosine receptors (A_1AR) within skeletal muscle has been shown to lower insulin sensitivity, thereby increasing the concentration of insulin needed to stimulate glucose and amino acid transport into muscle (Crist *et al.*, 1998). Increases in serum free fatty acids and TNF-α have also been observed to reduce skeletal muscle and hepatic insulin sensitivity in the *fa/fa* Zucker rat (Cheung *et al.*, 2000, Liu *et al.*, 2002). Sisk *et al.* (2001) showed only 2.5-fold higher fasting serum insulin concentrations in *fa/fa* Zucker rats compared to the lean Zucker rats. However, their study only lasted 5 weeks and measured serum insulin in 12-13 week old rats compared to our 8 week study that measured serum insulin in 14 week old rats. As well, they used a different insulin assay kit with possible varying sensitivity, and had a sample size of only n=4 rats per genotype. Ryder *et al.* (2001) showed 4.5-fold higher fasting serum insulin concentrations in 8 week old male ZDF rats compared to the lean rats. This study only lasted for 14 days, not allowing sufficient time for insulin resistance to worsen, possibly contributing to the lower difference between the obese and lean rat models. Houseknecht *et al.* (1998) showed approximately 13-fold higher fasting serum insulin concentrations in 8 week old male ZDF rats compared to the lean rats. This study was similar to Ryder *et al.* (2001) as both studies lasted 14 days and used the same strain of ZDF rats. This shows that possible inter-animal variability, which is impossible to control for, may be responsible for the slightly lower or higher differences between *fa/fa* and lean Zucker rats.

The *fa/fa* Zucker rats had a 6.5-fold higher fasting serum C-peptide concentration compared to lean Zucker rats (Figure 16). The reason why there was a smaller difference in C-peptide between genotypes compared to insulin may be due to the fact that that C-peptide is secreted in equimolar amounts with insulin. However, the liver extracts insulin, but not C-peptide. Therefore, C-peptide is an indicator of insulin secretion. Half-lives of insulin and C-peptide are also different, and excretion rates between genotypes may also be a factor.

The *fa/fa* rats fed the NCK diet had 52% lower fasting serum insulin and there was a trend toward lower fasting serum insulin concentrations in *fa/fa* rats fed 10,12, TOG, BIO, and NCK diets compared to 9,11, and in 10,12, TOG, and BIO diets compared to CTL (Figure 15). Ryder *et al.* (2001) similarly showed that ZDF rats fed 1.5% 50:50 CLA mixture of c9,t11 CLA and t10,c12 CLA had 23% lower fasting serum insulin compared to control. Houseknecht *et al.* (1998) and Nagao *et al.*, (2003) also showed that ZDF rats fed a 1.5% CLA mixture or a 1% CLA mixture, respectively, had a 46% and 65%, respectively, lower fasting serum insulin compared to control. Sisk *et al.* (2001) showed that *fa/fa* rats fed a 0.5% CLA mixture had a 36% lower fasting serum insulin concentration compared to control, but this was not statistically significant. Perhaps their sample size or methods of statistical analysis did not allow for significance to be reached. Despite not showing significance, our results also suggest that the c9,t11 CLA isomer is responsible for slightly increased concentrations of fasting serum insulin in the *fa/fa* Zucker rat. Similarly, Ryder *et al.* (2001) and Henriksen *et al.*

(2003) found that the c9,t11 CLA isomer negatively affected fasting serum insulin levels in the ZDF model. There is consensus amongst different studies that CLA mixtures tend to lower fasting serum insulin concentrations, while the c9,t11 CLA isomer seems to have little effect or may negatively increase fasting serum insulin concentrations (Ryder *et al.*, 2001; Henriksen *et al.*, 2003). However, the CLA diet did not normalize serum insulin levels in *fa/fa* rats to those of lean controls.

There was a trend (Figure 16) to lower fasting serum C-peptide concentrations in *fa/fa* rats fed 10,12, TOG, BIO, and NCK diets compared to 9,11 and CTL. This finding was expected since there is one molecule of insulin secreted for every molecule of C-peptide. Therefore, C-peptide is a marker of endogenous insulin secretion and should follow the same trends as seen with insulin, unless maladies such as insulinomas (tumors of the islet cells in the pancreas that can produce uncontrolled amounts of insulin and C-peptide) or hypokalemia (abnormally low level of potassium in the blood, leading to weakness and heart abnormalities, possibly resulting from diuretic intake, adrenal tumor, starvation, or other disorders) are present (Vezzosi *et al.*, 2003).

Oral Glucose Tolerance Test (OGTT)

Oral glucose tolerance testing was utilized as an *in vivo* test of insulin sensitivity. Serum glucose results from the OGTT showed a significant diet x

genotype x time interaction. However, the lean rats showed no difference by diet group in serum glucose concentrations at any time point, and were lower than all *fa/fa* groups at 15 minutes and 60 minutes. At time 0 minutes, the *fa/fa* rats fed 10,12 diet had a higher fasting serum glucose concentration than the other *fa/fa* rats except for BIO (Figure 17). This is in contrast with Henriksen *et al.*, (2003) who showed no differences amongst *fa/fa* Zucker CLA groups at time 0 minutes. However, these were female *fa/fa* Zucker rats and were administered the OGTT test after just 18 days on the CLA treatment diets.

At 15 minutes, the *fa/fa* rats fed 9,11, TOG, BIO and NCK had 14%-18% lower serum glucose concentrations compared to CTL, while the 10,12 group was not different from CTL or the other groups. Interestingly, Henriksen *et al.*, (2003) showed a trend for the t10,c12 CLA to have the lowest serum glucose for the *fa/fa* Zucker rats even compared to the c9,t11 CLA and 50:50 CLA mixture as well as control at 15 minutes. Once again, this study differed from ours in duration, gender and CLA diet compositions, possibly explaining these discrepancies.

At 30 minutes, the *fa/fa* rats fed the 10,12, TOG, BIO and NCK had 16%-33% lower serum glucose concentrations compared to 9,11 and CTL, while the 9,11 group was not different from CTL. Similarly, Henriksen *et al.* (2003) showed the *fa/fa* rats fed the 50:50 CLA mixture, and t10,c12 CLA diets had 14% and

23%, respectively, lower serum glucose concentrations compared to 9,11 and CTL at 30 minutes.

At 60 minutes, the *fa/fa* rats fed the 10,12, TOG, BIO and NCK had 21%-30% lower serum glucose concentrations compared to 9,11, while the 9,11 group had 12% lower serum glucose concentrations compared to CTL. Similarly, Ryder *et al.* (2001) showed that ZDF rats fed the 50:50 CLA mixture had the lowest serum glucose concentrations compared to c9,t11 CLA and control, while c9,t11 CLA was still lower than control at 60 minutes. Henriksen *et al.* (2003) showed that *fa/fa* Zucker rats fed the t10,c12 CLA had 13% lower serum glucose concentrations compared to CTL, however, the *fa/fa* Zucker rats fed the 50:50 CLA mixture and c9,t11 CLA only showed a trend to lower serum glucose concentrations compared to CTL. The similarity between our study and others is that the c9,t11 CLA isomer seems to have the least beneficial effect on glucose tolerance, while the t10,c12 CLA isomer individually or in combination with 2 or more CLA isomers has the greatest beneficial effect on glucose tolerance.

Area Under the Curve for Glucose (AUCg)

AUCg is calculated as the area under the curve for the serum glucose results during the oral glucose tolerance test, and it is a measure of the overall serum glucose response for the full duration of the oral glucose tolerance test. When the AUCg is smaller, it represents a more favorable glucose response for

the full duration of the oral glucose tolerance test. The *fa/fa* Zucker rats had a 1.4-fold larger AUCg compared to the lean Zucker rats.

The *fa/fa* rats fed the TOG, BIO and NCK diets had lower (24-29%) AUCg compared to CTL, with a concomitant trend to a smaller AUCg in *fa/fa* rats fed TOG, BIO, and NCK diets compared to 9,11 and in the 10,12 diet compared to 9,11 and CTL (Figure 18). Similarly, Henriksen *et al.* (2003) found that 11-12 week old female *fa/fa* Zucker rats fed 1.5 g total CLA/kg body weight of 50:50 CLA mixture or t10,c12 CLA isomer for 3 weeks exhibited a decrease in AUCg of 10% and 16%, respectively, with the c9,t11 CLA isomer having little effect on AUCg compared to control or the other CLA isomers or mixture. The Henriksen *et al.* (2003) results for the 50:50 CLA mixtures concur with our findings, albeit our findings show a trend that CLA in a mixture form has more of a beneficial effect on AUCg, than does the t10,c12 CLA individual isomer as Henriksen *et al.* (2003) observed. However, we fed a much smaller amount of the t10,c12 CLA isomer than did Henriksen *et al.* (2003), which may have had an impact on the results. Our study used 0.4% t10,c12 CLA isomer which amounted to approximately 0.1 g t10,c12 CLA per day for the 10,12 *fa/fa* rat diet group. Henriksen *et al.* (2003), used 1.5 g total CLA/kg body weight which amounted to approximately 0.4 g t10,c12 CLA per day, that was steadily increased according to the weight of the rats over the 3 week study. Another possible reason for the difference is that the effects of the t10,c12 CLA isomer may be duration

dependent and the beneficial effect that Henriksen *et al.* (2003) showed may have diminished if the study was prolonged.

Homeostasis Model Assessment Index of Insulin Resistance (HOMA-IR)

Insulin resistance was estimated by calculating the HOMA-IR index [fasting serum insulin (uU/ml) x fasting plasma glucose (mmol/L)/22.5] (Katsuki A *et al.*, 2001). The *fa/fa* Zucker rats had a 14-fold higher estimated insulin resistance compared to the lean Zucker rats (Figure 19). The *fa/fa* Zucker rats carry the *fa* gene for obesity. The *fa* gene leads to a deficiency in the leptin receptors, that causes hyperphagia and the high amounts of circulating leptin is positively correlated with obesity and the development of early-onset severe hyperinsulinemia due to insulin resistant peripheral tissues (Terrettaz *et al.*, 1983). Thus, the *fa/fa* Zucker rat represents a good model for examining insulin resistance.

There was a significant CLA diet effect such that Zucker rats fed the 10,12, TOG, BIO and NCK had lower (34-50%) estimated insulin resistance compared to 9,11, while the TOG and NCK diets had 44% and 48%, respectively, lower estimated insulin resistance compared to CTL, regardless of genotype (Figure 20). There was also a concomitant trend (Figure 19) for lower estimated insulin resistance in *fa/fa* rats fed 10,12, TOG, BIO, and NCK diets compared to 9,11 and CTL. However, CLA diet treatment did not normalize

estimated insulin resistance in the *fa/fa* Zucker rats to levels observed in lean controls. Tricon *et al.* (2004) found that supplementing healthy male human volunteers with 0.59, 1.19, or 2.38 g/d of impure c9,t11 CLA and 0.63, 1.26, or 2.52 g/d of impure t10,c12 CLA, respectively, had no effects on estimated insulin resistance. Our results agree that the c9,t11 CLA isomer had either no effect or possibly a slight negative effect on estimated insulin resistance. However, there was a trend (Figure 19) for the t10,c12 CLA isomer to lower estimated insulin resistance compared to CTL despite not being significant. Conversely, our 2 and 4 CLA isomer mixtures proved to have dramatic results on lowering estimated insulin resistance compared to CTL. Our results would seem to indicate that the t10,c12 CLA isomer lowers estimated insulin resistance, however, a CLA mixture (with a greater total amount of CLA) seems to have a more significant impact on estimated insulin resistance regardless of genotype. It is also possible that the presence of other CLA isomers such as the t8,c10 CLA or c11,t13 CLA found in the 4 CLA isomer mixture may have beneficial effects either singly, or synergistically with t10,c12. While our data remain inconclusive on this issue, the actions of other isomers cannot yet be discounted.

Interestingly, the diets that reduced insulin resistance also produced a marked increase in visceral fat relative to body weight, although there was no overall change in body weight. Normally insulin resistance is accompanied by higher amounts of visceral fat. A rationale for this observation may be linked to remodeling of the adipocytes: cell size becomes smaller although there is a

concomitant increase in adipocyte numbers. These changes have been shown to allow the adipose tissue to store more lipids, thus improving insulin resistance (de Souza *et al.*, 2001). Similarly, Noto *et al.* (2004, 2005) showed that the administration of a CLA mixture improved OGTT in *fa/fa* Zucker rats with a concomitant decrease in adipocyte cell size.

Serum Triglycerides and Free Fatty Acids

The *fa/fa* Zucker rats had a 7.3-fold higher serum triglyceride concentration and a 2.5-fold greater serum free fatty acid concentration compared to the lean Zucker rats (Figures 21 & 22). Similarly, Ryder *et al.* (2001) showed a 4.7-fold higher serum triglyceride concentration and a 5.4-fold higher serum free fatty acid concentration in male ZDF rats compared to lean Zucker rats. These differences in serum triglyceride and serum free fatty acid concentrations between our study and Ryder *et al.* (2001) can probably be explained by species strain variability, which is impossible to control for, and may be responsible for slightly higher differences between male ZDF rats and male *fa/fa* Zucker rats compared to lean Zucker rats.

In our study, there was no effect of dietary CLA treatment on serum triglycerides. Similarly, Sisk *et al.* (2001) found that a 0.5% CLA mixture had no effect on serum triglycerides in male *fa/fa* Zucker rats. Conversely, Ryder *et al.* (2001) observed that in male ZDF rats fed the 1.5% c9,t11 CLA and a 1.5%

50:50 CLA mixture, serum triglyceride concentrations were reduced by 20% and 30%, respectively. It is not the percentage or amount of CLA dosage that caused the difference, since our NCK diet contained a 1.9% CLA mixture. Therefore, the differences seen may be due to study duration and age of rats. The Ryder *et al.* (2001) study only lasted 14 days with 8 week old rats, while the study by Sisk *et al.* (2001) lasted 5 weeks with 12-13 week old rats and ours lasted 8 weeks with 14 week old rats at the end of the study. Alternatively, our study had exhibited a large variability in this parameter, a factor that could have accounted for the failure to reach statistical significance.

Our study also observed no effect of dietary CLA treatment on serum free fatty acids. In contrast, Houseknect *et al.* (1998) observed lower serum free fatty acid levels compared to controls in male ZDF rats fed a 1.5% CLA mixture for 14 days. Henriksen *et al.* (2003) showed that female *fa/fa* Zucker rats fed a 1.5 g/kg body weight 50:50 CLA mixture or t10,c12 CLA had 14% and 27%, respectively, lower serum free fatty acid levels compared to control, however, c9,t11 CLA had no effect. Ryder *et al.* (2001) showed similar findings where male ZDF rats fed a 1.5% 50:50 mixture had serum free fatty acid concentrations decrease 21%, while the 1.5% c9,t11 CLA had no effect. The resounding difference between these three studies and our own was the duration of the experiment. Ours lasted 8 weeks, which can be considered a relatively long-term study compared to the Houseknect *et al.* (1998) 14 day study, the Ryder *et al.* (2001) 14 day study and the Henriksen *et al.* (2003) 21 day study. Whether this is the reason for the

difference between our studies is undetermined. The strain and gender of the rats administered the differing CLA diets may have also had an impact on the serum free fatty acid levels.

Skeletal Muscle AMPK-α and phospho-AMPK-α

No studies have examined the direct effects of dietary CLA supplementation on the levels of AMPK within skeletal muscle. Since AMPK has been shown in previous studies to mediate the action of antidiabetic drugs such as metformin and rosiglitazone (Musi *et al.*, 2002; Fryer *et al.*, 2002), it leads to the proposed hypothesis that AMPK activators may function as antidiabetic agents. CLA has been shown to enhance adiponectin levels in adipose tissue (Noto *et al.*, 2004) as well as plasma in the Zucker *fa/fa* rat model (Nagao *et al.*, 2003), and plasma adiponectin was recently shown to activate AMPK-α within skeletal muscle (Tomas *et al.*, 2004). Since serum adiponectin levels were similarly elevated in response to CLA during the course of our study as shown by Zirk *et al.* (2005) and previously observed by Noto *et al.* (2004), we hypothesized that CLA may improve insulin sensitivity via skeletal muscle AMPK-α, through the increase of serum adiponectin levels. However, to our surprise there was no significant effect of genotype or dietary CLA on skeletal muscle AMPK-α or phospho-AMPK-α levels (Figures 23 & 24), or the ratio of skeletal muscle phospho-AMPK-α / AMPK-α (Figure 25). Our study suggests that despite elevated serum adiponectin levels observed in these same animals by Zirk *et al.*

(2005) and a reduction of insulin resistance through improved glucose tolerance in response to dietary CLA, these improvements are not coupled to changes in skeletal muscle AMPK-α protein levels or activation.

Skeletal Muscle TNF-α

Higher TNF-α levels are a characteristic of the metabolic syndrome and there is speculation that TNF-α is involved in the pathogenesis of Type 2 diabetes mellitus and insulin resistance (Nilsson *et al.*, 1998). Consequently, we considered TNF-α to be possible target for CLA action. TNF-α has two forms and is present both as a membrane bound protein (26 kDa) and a physiologically active (19 kDa) form (Pallandino *et al.*, 2003). Although TNF-α may be a mediator of obesity-linked insulin resistance and has been proven to elicit insulin resistance in adipose tissue, it was still inconclusive as to whether the same effect would be seen in skeletal muscle tissue, and whether dietary CLA would reduce TNF-α levels in skeletal muscle.

Our results showed a surprising finding where the skeletal muscle of *fa/fa* Zucker rats had a 1.4-fold lower active TNF-α (19 kDa) and a 1.8-fold lower latent TNF-α (26 kDa) compared to the lean Zucker rats (Figures 26 & 27). Consequently, we observed a 1.6-fold lower total skeletal muscle TNF-α level (19 kDa + 26 kDa) compared to the lean Zucker rats (Figure 28). No studies to our knowledge have reported TNF-α protein levels in skeletal muscles of *fa/fa* and

lean Zucker rats. Hotamisligil *et al.* (1995) found 2-fold more TNF-α protein levels in adipose tissue from 19 obese pre-menopausal women relative to 18 normal weight controls. This is the result we expected to see, however, we observed that the lean rats had higher levels of TNF-α protein in skeletal muscle compared to the obese *fa/fa* Zucker rats.

Another finding of our study was the lack of effect of dietary CLA treatment on skeletal muscle TNF-α (19 kDa), TNF-α (26 kDa) or total skeletal muscle TNF-α (19 kDa + 26 kDa) levels. In contrast, Tsuboyama-Kasaoka *et al.* (2000) found that female C57BL/6J mice fed a CLA mixture (1% wt/wt), which resulted in insulin resistance was coupled with 88% higher TNF-α mRNA levels in white adipose tissue, and a surprising ~50% lower TNF-α mRNA levels in skeletal muscle compared to control. Whether the differences seen were due to the species, or that Tsuboyama-Kasaoka *et al.* (2000) examined TNF-α mRNA expression compared to TNF-α protein cannot be determined at this point.

There was a trend (Figures 26, 27 & 28) for higher skeletal muscle TNF-α (19 kDa), TNF-α (26 kDa) and total TNF-α (19 kDa + 26 kDa) levels in lean rats fed TOG diet compared to CTL. This shows that the 50:50 CLA mixture of c9,t11 CLA and t10,c12 CLA elevated skeletal muscle TNF-α within the lean genotype. Interestingly, despite lean rats fed the TOG diet having higher amounts of TNF-α, there were no negative effects on insulin resistance, glucose tolerance, or circulating FFA. Since higher amounts of TNF-α normally occur in conjunction

with apoptosis of adipocytes (Tsuboyama-Kasaoka *et al.*, 2000), less adipose tissue would have been expected in our lean rats with elevated levels of TNF-α. This would have resulted in a concomitant increase in serum FFA inhibiting insulin-stimulated glucose uptake causing peripheral tissue insulin resistance, yet none of these traits were exhibited in the lean rats. In fact, the CLA-fed lean rats, with the exception of the 9,11 group, showed improved glucose tolerance and estimated insulin resistance even though significance was not achieved. The reason for unaltered insulin sensitivity in the lean rats despite increased levels of TNF-α is undetermined and cannot be explained at this time. However, it is possible that the elevated levels of TNF-α protein within the lean Zucker rats actually stimulated glucose uptake in skeletal muscle. Yamasaki *et al.* (1996) observed that incubation of rat L6 myoblasts, which contain receptors for TNF-α, for 48 hours with TNF-α weakly stimulated glucose uptake by 3-fold. Similarly, Ciaraldi *et al.* (1998) observed that an acute 90-min exposure to TNF-α stimulated glucose uptake to a greater extent than did insulin in cultured human muscle cells from non-diabetic subjects. When these same cells from non-diabetic subjects were continually exposed to TNF-α for a duration of 24 hours, it resulted in a further stimulation of glucose uptake. After chronic TNF-α exposure, the level of GLUT1 protein was elevated 4.6-fold in cells from non-diabetic subjects. The reason for unaltered insulin sensitivity in the lean rats despite increased levels of TNF-α in our study still remains inconclusive.

Interestingly, the *fa/fa* Zucker rats had a 1.1-fold higher skeletal muscle TNF-α ratio (19 kDa/(19 kDa + 26 kDa)) compared to the lean Zucker rats (Figure 29), but there was no effect of dietary CLA treatment. Even though the *fa/fa* rats had lower total TNF-α levels (19 kDa + 26 kDa), the percentage of active TNF-α (19 kDa) compared to the total amount of TNF-α (19 kDa + 26 kDa) was higher in the *fa/fa* Zucker rats compared to the lean rats. This suggests that the *fa/fa* Zucker rats have more active TNF-α (19 kDa) levels compared to membrane bound skeletal muscle TNF-α (26 kDa) levels in comparison to the lean Zucker rats. Concomitantly, these results show us that the percentage of active TNF-α (19 kDa) compared to total TNF-α (19 kDa + 26 kDa) levels was not affected by dietary CLA in either the *fa/fa* or the lean Zucker rats.

In conclusion, our study shows that CLA does not have an overall diet effect in both genotypes, rather diet differences are seen in the *fa/fa* group alone. We have demonstrated that CLA mixtures as well as the t10,c12 CLA isomer can elicit a beneficial metabolic response in the *fa/fa* Zucker rat, a rodent model of insulin resistance, glucose intolerance, hyperinsulinemia, hyperlipidemia, and central obesity. The metabolic improvement due to the CLA mixtures and the t10,c12 CLA isomer was a reduction of insulin resistance via improved glucose tolerance within the *fa/fa* Zucker rat, while the c9,t11 CLA isomer had no effect. However, the improvements seen with the CLA mixtures and t10,c12 CLA isomer were not coupled to changes in skeletal muscle AMPK-α protein levels or activation. As well, the improvements seen with the CLA mixtures and t10,c12

CLA isomer were not coupled to changes in hyperlipidemia reduction or TNF-α protein reduction in skeletal muscle or weight gain. Therefore, another mechanism(s) must mediate CLA's effects on improved oral glucose tolerance and insulin resistance. It is possible that CLA may affect glucose transporters such as GLUT4 and GLUT1 in skeletal muscle, or activate AMPK-α in varying other tissues, such as adipose or liver leading to reduced insulin resistance, however, more examination is required to elucidate these possible mechanisms.

6. SUMMARY AND CONCLUSIONS

Major Research Findings

Adipose Tissue Mass

- Visceral fat pad weights (absolute amount and relative to body weight) were higher in *fa/fa* rats fed the t10,c12 CLA isomer individually or in combination with other CLA isomers, despite there being no changes in body weight.
- Visceral fat pad weights (absolute amount and relative to body weight) were not affected in the *fa/fa* rats fed the c9,t11 CLA isomer.

Serum Insulin

- Fasting serum insulin concentrations were significantly lower in the *fa/fa* rats fed the 4 CLA isomer mixture diet.
- There was a trend towards lower fasting serum insulin concentrations in *fa/fa* rats fed the t10,c12 CLA isomer individually and in combination with other CLA isomers. However, fasting serum insulin concentrations were not altered by the c9,t11 CLA isomer.

Oral Glucose Tolerance and Homeostasis Model Assessment Index of Insulin Resistance (HOMA-IR)

- Oral glucose tolerance and HOMA-IR were significantly improved in the *fa/fa* rats fed the t10,c12 CLA isomer individually or in combination with c9,t11 CLA and/or other CLA isomers.
- The c9,t11 CLA isomer had the least beneficial effect on oral glucose tolerance with no effect on HOMA-IR in the *fa/fa* rats.

Serum Triglycerides and Free Fatty Acids

- Serum triglycerides and serum free fatty acid concentrations were elevated in the *fa/fa* genotype, but were not altered by dietary CLA.

Skeletal Muscle AMPK-α and phospho-AMPK-α

- Skeletal muscle AMPK-α and skeletal muscle phospho-AMPK-α levels were not affected by genotype or dietary CLA.

Skeletal Muscle TNF-α

- Skeletal muscle TNF-α levels were higher in lean rats compared to *fa/fa* rats.
- Skeletal muscle TNF-α levels were not affected by dietary CLA, however, there was a trend towards higher TNF-α levels in lean rats fed the CLA as a 50:50 mixture of c9,t11 CLA and t10,c12 CLA.

Strengths and Limitations

Strengths

- The 8 week duration of our study is considered relatively long term in comparison to other studies involving supplementation with dietary CLA.
- Our sample size of n=10 male lean and n=10 obese *fa/fa* Zucker rats was adequate.
- We used purified preparations of c9,t11 CLA and t10,c12 CLA individually, and in combination with other CLA isomers (purified 50:50 mixture with c9,t11 CLA and/or a two or four isomer CLA mixture).
- Multiple parameters and well accepted, proven techniques were applied to elucidate our hypothesis.
- Results were compiled and analyzed using two-way Analysis of Variance (ANOVA) for the main effects (genotype, diet, and interactions of genotype x diet), with significance set at $P<0.05$.
- Pair weighed groups were used in the experimental design to maintain body weights equivalent to the lightest *fa/fa* or lean Zucker rats, thus ensuring differences in insulin sensitivity were not due to differences in body weight.

Limitations

- We only studied male *fa/fa* and lean Zucker rats.
- CLA isomer products were in free fatty acid form, while CLA in food is found in the triacylglycerol form.
- Soybean oil was used as the base lipid in the diet and may have had an impact on the results, due to its elevated content of polyunsaturatesd fatty acids and more balanced ratio of omega-3:omega-6 (n-3:n-6) fatty acids in relation to other oils.
- We only observed final body weight and adipose weight, and did not look at overall body composition.
- We only studied gastrocnemius skeletal muscle, while differing effects may have been seen in other samples of skeletal muscle.
- Insulin sensitivity was measured using HOMA-IR index [fasting serum insulin (uU/ml) x fasting plasma glucose (mmol/L)/22.5], which is an estimated parameter.
- Western results for skeletal muscle AMPK-α, phospho-AMPK-α and TNF-α (19 kDa, 26 kDa) were unpredictable and not easily obtained. This resulted in a decreased sample size, possibly contributing to significance not being reached for these parameters.

Future Directions

- Further investigation is required in a variety of obese models that are at risk for Syndrome X to determine if CLA actually has a beneficial role in attenuating insulin resistance and glucose intolerance.
- The mechanism(s) by which CLA isomers individually and in combination influence the metabolic risk factors such as glucose intolerance and insulin resistance found in Syndrome X need to be further explored and confirmed.
- Commercial CLA products are mainly present in the free fatty acid form, whereas dietary sources of CLA isomers are mainly in the triacylglycerol form. Differences in overall metabolism of CLA isomers in the free fatty acid form vs. triacylglycerol form could influence the mechanisms of CLA within the body.
- Long term studies are required to establish if dietary CLA supplementation has either a dose or duration effect on toxicity to assure safety.
- Studies must be done with a variety of protein-based diets supplemented with CLA isomers individually and in combination, to determine whether it is the dietary CLA, the protein base, or a synergistic effect of the combination of the dietary CLA and the protein base in question that is having the beneficial effects.
- Investigating varying dietary percentages of c9,t11 CLA and t10,c12 CLA individually and elucidating the optimal ratios and dietary percentages of

the combination of c9,t11 CLA and t10,c12 CLA to modulate the positive and negative effects.

- Investigating if dietary CLA both individually and in combination have an effect on AMPK-α levels and activation in varying tissues such as brown adipose, white adipose or additional muscle tissues. Studying different species, genotypes and genders is required.
- Investigating if dietary CLA both individually and in combination have an effect on TNF-α levels in varying tissues such as brown adipose, white adipose or additional muscle tissues. Studying different species and genotypes is required.
- Determining whether elevated serum adiponectin concentrations induced by dietary CLA supplementation affect AMPK-α levels in tissues other than skeletal muscle in different species and genders, with a concomitant improvement to insulin sensitivity.
- Investigating CLA's role both individually and in combination upon skeletal muscle GLUT1 and GLUT4 expression, plus localization in varying hyperinsulinemic subjects.
- The ultimate objective is to determine the efficacy of dietary CLA in the clinical treatment of obesity and Type 2 diabetes.

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APPENDIX- RAW DATA FOR FIGURES 1-29

Table 1 - The effect of 9,11, 10,12, TOG, BIO, NCK and CTL diets on final body weight in Zucker rats[1].

Body Weight (g)	9,11[2]	10,12[2]	TOG[2]	BIO[2]	NCK[2]	CTL[2]	Genotype Mean[3]
fa/fa	543 ± 13	545 ± 10	567 ± 18	534 ± 10	534 ± 14	561 ± 13	547 ± 5◆
Lean	336 ± 7	332 ± 9	326 ± 7	325 ± 8	317± 6	328 ± 5	327 ± 3◆

[1]Raw data for Figure 1
[2] Values are means±SEM, n=10
[3] Values are means±SEM, n=60
◆ Significant genotype effect (P<0.0001)

Table 2 - The effect of 9,11, 10,12, TOG, BIO, NCK and CTL diets on total feed intake in Zucker rats [1,2].

Total Feed Intake (g)	9,11[3]	10,12[3]	TOG[3]	BIO[3]	NCK[3]	CTL[3]
fa/fa	1563 ± 55	1399 ± 30*	1539 ± 50	1516 ± 32	1466 ± 35	1666 ± 51
Lean	959 ± 21	999 ± 17	1023 ± 23	976 ± 35	901 ± 17	1022 ± 24

[1]Raw data for Figure 2
[2]Significant diet x genotype interaction (P=0.0211)
[3]Values are means±SEM, n=10
* Significant pre-determined contrast (P<0.05) between a treatment group and CTL

Table 3 - The effect of 9,11, 10,12, TOG, BIO, NCK and CTL diets on feed intake per day in Zucker rats [1,2].

Total Feed Intake per day (g)	9,11[3]	10,12[3]	TOG[3]	BIO[3]	NCK[3]	CTL[3]
fa/fa	27.9 ± 1.0	25.0 ± 0.5*	27.5 ± 0.9	27.1 ± 0.6	26.2 ± 0.6	29.7 ± 0.9
Lean	17.1 ± 0.4	17.8 ± 0.3	18.3 ± 0.4	17.4 ± 0.6	16.1 ± 0.3	18.3 ± 0.4

[1]Raw data for Figure 3
[2]Significant diet x genotype interaction (P=0.0206)
[3]Values are means±SEM, n=10
* Significant pre-determined contrast (P<0.05) between a treatment group and CTL

Table 4 - The effect of 9,11, 10,12, TOG, BIO, NCK and CTL diets on feed efficiency ratio (FER) in Zucker rats [1,2].

FER	9,11[3]	10,12[3]	TOG[3]	BIO[3]	NCK[3]	CTL[3]
fa/fa	0.187 ± 0.005[#]	0.211 ± 0.009	0.209 ± 0.005*	0.193 ± 0.006[#]	0.199 ± 0.006[#]	0.182 ± 0.003
Lean	0.161 ± 0.003	0.154 ± 0.004	0.143 ± 0.006	0.155 ± 0.006	0.166 ± 0.006	0.155 ± 0.002

[1]Raw data for Figure 4
[2]Significant diet x genotype interaction (P=0.0206)
[3]Values are means±SEM, n=10
* Significant pre-determined contrast (P<0.05) between a treatment group and CTL
# Significant pre-determined contrast (P<0.05) between a treatment group and TOG

Table 5 - The effect of 9,11, 10,12, TOG, BIO, NCK and CTL diets on epididymal fat pad weight in Zucker rats [1].

Epididymal Fat Pad Weight (g)	9,11[2]	10,12[2]	TOG[2]	BIO[2]	NCK[2]	CTL[2]	Genotype Mean[3]
fa/fa	16.0 ± 0.6	19.2 ± 0.9	21.9 ± 0.8	20.0 ± 0.6	20.4 ± 0.8	17.8 ± 1.2	19.2 ± 0.4◆
Lean	5.2 ± 0.5	6.0 ± 0.3	6.1 ± 0.5	5.3 ± 0.3	5.1 ± 0.3	4.7 ± 0.3	5.4 ± 0.2◆

[1]Raw data for Figure 5
[2] Values are means±SEM, n=10
[3] Values are means±SEM, n=60
◆ Significant genotype effect (P<0.0001)

Table 6 - The effect of 9,11, 10,12, TOG, BIO, NCK and CTL diets on epididymal fat pad weight in Zucker rats [1,2].

Diet Groups	Means (g)
9,11[3]	10.6 ± 1.3^{c}
10,12[3]	12.6 ± 1.6^{ab}
TOG[3]	14.0 ± 1.9^{a}
BIO[3]	12.6 ± 1.7^{ab}
NCK[3]	12.7 ± 1.8^{bc}
CTL[3]	11.2 ± 1.6^{c}

[1]Raw data for Figure 6
[2]Significant diet effect ($P<0.0001$)
[3]Values are means±SEM, n=20. Means with different letters are significantly different ($P<0.05$), while means with the same letter are not different ($P>0.05$) by Duncan's Multiple Range test.

Table 7 - The effect of 9,11, 10,12, TOG, BIO, NCK and CTL diets on perirenal fat pad weight in Zucker rats [1].

Perirenal Fat Pad Weight (g)	9,11[2]	10,12[2]	TOG[2]	BIO[2]	NCK[2]	CTL[2]	Genotype Mean[3]
fa/fa	23.7 ± 1.1	28.4 ± 1.2	31.3 ± 2.2	28.0 ± 1.3	28.9 ± 1.3	23.9 ± 1.6	27.4 ± 0.7◆
Lean	5.2 ± 0.6	5.2 ± 0.6	5.1 ± 0.6	4.2 ± 0.3	3.9 ± 0.4	4.7 ± 0.3	4.7 ± 0.2◆

[1]Raw data for Figure 7
[2] Values are means±SEM, n=10
[3] Values are means±SEM, n=60
◆ Significant genotype effect (P<0.0001)

Table 8 - The effect of 9,11, 10,12, TOG, BIO, NCK and CTL diets on visceral fat pad weight in Zucker rats [1].

Visceral Fat Pad Weight (g)	9,11[2]	10,12[2]	TOG[2]	BIO[2]	NCK[2]	CTL[2]	Genotype Mean[3]
fa/fa	39.7 ± 1.4	47.6 ± 2.0	53.2 ± 2.9	48.0 ± 1.7	49.3 ± 1.9	41.7 ± 2.3	46.6 ± 1.0◆
Lean	10.5 ± 1.0	11.2 ± 0.8	11.2 ± 1.0	9.5 ± 0.6	8.9 ± 0.6	9.4 ± 0.6	10.1 ± 0.3◆

[1] Raw data for Figure 8
[2] Values are means±SEM, n=10
[3] Values are means±SEM, n=60
◆ Significant genotype effect (P<0.0001)

Table 9 - The effect of 9,11, 10,12, TOG, BIO, NCK and CTL diets on visceral fat pad weight in Zucker rats [1,2].

Diet Groups	Means (g)
9,11[3]	25.1 ± 3.5^{c}
10,12[3]	29.4 ± 4.3^{ab}
TOG[3]	32.2 ± 5.0^{a}
BIO[3]	28.8 ± 4.5^{abc}
NCK[3]	29.1 ± 4.7^{bc}
CTL[3]	25.5 ± 3.9^{c}

[1]Raw data for Figure 9
[2]Significant diet effect (P=0.01)
[3] Values are means±SEM, n=20. Means with different letters are significantly different (P<0.05), while means with the same letter are not different (P>0.05) by Duncan's Multiple Range test.

Table 10 - The effect of 9,11, 10,12, TOG, BIO, NCK and CTL diets on epididymal fat pad weight relative to body weight in Zucker rats [1,2].

Epidiymal Fat Pad Weight Relative to Body Weight (g/100 g)	9,11[3]	10,12[3]	TOG[3]	BIO[3]	NCK[3]	CTL[3]
fa/fa	2.95 ± 0.08[#]	3.51 ± 0.13	3.87 ± 0.12	3.74 ± 0.07	3.82 ± 0.11	3.16 ± 0.17
Lean	1.55 ± 0.12	1.80 ± 0.04	1.87 ± 0.11	1.62 ± 0.06	1.60 ± 0.10	1.42 ± 0.07

[1]Raw data for Figure 10
[2]Significant diet x genotype interaction (P=0.0013)
[3]Values are means±SEM, n=10
[#] Significant pre-determined contrast (P<0.05) between a treatment group and TOG

Table 11 - The effect of 9,11, 10,12, TOG, BIO, NCK and CTL diets on perirenal fat pad weight relative to body weight in Zucker rats[1,2].

Perirenal Fat Pad Weight Relative to Body Weight (g/100 g)	9,11[3]	10,12[3]	TOG[3]	BIO[3]	NCK[3]	CTL[3]
fa/fa	4.36 ± 0.15[#]	5.19 ± 0.18	5.47 ± 0.27*	5.23 ± 0.18*	5.41 ± 0.16*	4.24 ± 0.22
Lean	1.54 ± 0.16	1.56 ± 0.14	1.56 ± 0.16	1.29 ± 0.08	1.22 ± 0.11	1.42 ± 0.08

[1]Raw data for Figure 11
[2]Significant diet x genotype interaction (P<0.0001)
[3]Values are means±SEM, n=10
* Significant pre-determined contrast (P<0.05) between a treatment group and CTL
# Significant pre-determined contrast (P<0.05) between a treatment group and TOG

Table 12 - The effect of 9,11, 10,12, TOG, BIO, NCK and CTL diets on visceral fat pad weight relative to body weight in Zucker rats [1,2].

Visceral Fat Pad Weight Relative to Body Weight (g/100 g)	9,11[3]	10,12[3]	TOG[3]	BIO[3]	NCK[3]	CTL[3]
fa/fa	4.36 ± 0.15[#]	5.19 ± 0.18	5.47 ± 0.27*	5.23 ± 0.18*	5.41 ± 0.16*	4.24 ± 0.22
Lean	3.09 ± 0.26	3.36 ± 0.17	3.42 ± 0.26	2.91 ± 0.12	2.82 ± 0.19	2.84 ± 0.14

[1]Raw data for Figure 12
[2]Significant diet x genotype interaction (P<0.0001)
[3]Values are means±SEM, n=10
* Significant pre-determined contrast (P<0.05) between a treatment group and CTL
# Significant pre-determined contrast (P<0.05) between a treatment group and TOG

Table 13 - The effect of 9,11, 10,12, TOG, BIO, NCK and CTL diets on epididymal / visceral fat pad ratio in Zucker rats [1,2].

Epididymal / Visceral Fat Pad Ratio	9,11[3]	10,12[3]	TOG[3]	BIO[3]	NCK[3]	CTL[3]
fa/fa	0.405 ± 0.010	0.403 ± 0.008	0.417 ± 0.012	0.418 ± 0.010	0.414 ± 0.008	0.428 ± 0.018
Lean	0.507 ± 0.014	0.543 ± 0.019	0.552 ± 0.017	0.560 ± 0.011	0.573 ± 0.018*	0.500 ± 0.011

[1]Raw data for Figure 13
[2]Significant diet x genotype interaction (P<0.0232)
[3]Values are means±SEM, n=10
* Significant pre-determined contrast (P<0.05) between a treatment group and CTL

Table 14 - The effect of 9,11, 10,12, TOG, BIO, NCK and CTL diets on perirenal / visceral fat pad ratio in Zucker rats [1,2].

Perirenal / Visceral Fat Pad Ratio	9,11[3]	10,12[3]	TOG[3]	BIO[3]	NCK[3]	CTL[3]
fa/fa	0.595 ± 0.010	0.597 ± 0.008	0.583 ± 0.012	0.582 ± 0.010	0.586 ± 0.008	0.572 ± 0.018
Lean	0.493 ± 0.014	0.457 ± 0.019	0.448 ± 0.017	0.440 ± 0.011	0.427 ± 0.018*	0.500 ± 0.011

[1]Raw data for Figure 14
[2]Significant diet x genotype interaction (P=0.0229)
[3]Values are means±SEM, n=10
* Significant pre-determined contrast (P<0.05) between a treatment group and CTL

Table 15 - The effect of 9,11, 10,12, TOG, BIO, NCK and CTL diets on fasting serum insulin concentrations in Zucker rats [1,2].

Serum Insulin (pmol/L)	9,11[3]	10,12[3]	TOG[3]	BIO[3]	NCK[3]	CTL[3]
fa/fa	4138 ± 454	2255 ± 155	2104 ± 218	2455 ± 183	1935 ± 162*	3991 ± 597
Lean	270 ± 47	228 ± 30	230 ± 25	217 ± 32	257 ± 31	243 ± 30

[1]Raw data for Figure 15
[2]Significant diet x genotype interaction (P=0.0108)
[3]Values are means±SEM, n=10
* Significant pre-determined contrast (P<0.05) between a treatment group and CTL

Table 16 - The effect of 9,11, 10,12, TOG, BIO, NCK and CTL diets on fasting serum C-peptide concentrations in Zucker rats [1].

Serum C-peptide (pmol/L)	9,11[2]	10,12[2]	TOG[2]	BIO[2]	NCK[2]	CTL[2]	Genotype Mean[3]
fa/fa	10440 ± 1237	7403 ± 653	6899 ± 848	8164 ± 1051	6917 ± 638	9902 ± 707	8287 ± 391◆
Lean	1153 ± 571	1077 ± 205	1111 ± 130	1057 ± 161	1129 ± 130	1069 ± 100	1099 ± 60◆

[1]Raw data for Figure 16
[2] Values are means±SEM, n=10
[3] Values are means±SEM, n=60
◆ Significant genotype effect (P<0.0001)

Table 17 - The effect of 9,11, 10,12, TOG, BIO, NCK and CTL diets on serum glucose concentrations during oral glucose tolerance testing (OGTT) in Zucker rats [1,2].

OGTT (mmol/L)	9,11[3]	10,12[3]	TOG[3]	BIO[3]	NCK[3]	CTL[3]
fa/fa						
0 minutes	9.09 ± 0.37^{bc}	10.54 ± 0.66^{a}	8.98 ± 0.22^{bc}	9.34 ± 0.35^{ab}	9.10 ± 0.41^{bc}	9.06 ± 0.76^{bc}
15 minutes	14.88 ± 0.84^{b}	15.67 ± 0.92^{ab}	14.37 ± 0.67^{b}	14.16 ± 0.63^{b}	14.30 ± 0.79^{b}	17.43 ± 1.11^{a}
30 minutes	17.46 ± 1.01^{a}	14.66 ± 1.26^{b}	13.30 ± 0.73^{bc}	13.49 ± 0.72^{b}	12.48 ± 0.61^{bcd}	18.62 ± 1.09^{a}
60 minutes	18.27 ± 1.30^{b}	13.67 ± 1.03^{c}	13.45 ± 0.72^{c}	14.49 ± 0.81^{c}	12.80 ± 0.66^{c}	20.73 ± 1.21^{a}
Lean						
0 minutes	7.99 ± 0.43^{bcd}	7.65 ± 0.46^{d}	7.13± 0.28^{d}	7.83 ± 0.30^{cd}	6.96 ± 0.36^{d}	7.60 ± 0.24^{d}
15 minutes	11.06 ± 0.69^{c}	10.05 ± 0.48^{c}	10.79 ± 0.72^{c}	10.49 ± 0.40^{c}	10.52 ± 0.68^{c}	11.15 ± 0.55^{c}
30 minutes	10.54 ± 0.58^{d}	10.61 ± 0.38^{d}	11.06 ± 0.85^{cd}	10.72 ± 0.27^{d}	10.86 ± 0.58^{d}	10.77 ± 0.54^{d}
60 minutes	9.88 ± 0.51^{d}	9.78 ± 0.56^{d}	10.12 ± 0.51^{d}	10.35 ± 0.48^{d}	8.79 ± 0.52^{d}	10.36 ± 0.44^{d}

[1]Raw data for Figure 17
[2]Significant diet x genotype x time interaction (P<0.0001)
[3]Values are means±SEM, n=10. Means with different letters are significantly different (P<0.05), while means with the same letter are not different (P>0.05) by Duncan's Multiple Range test.

Table 18 - The effect of 9,11, 10,12, TOG, BIO, NCK and CTL diets on area under the curve for glucose (AUCg) in Zucker rats [1,2].

AUCg ($mmol \cdot min^{-1} \cdot L^{-1}$)	9,11[3]	10,12[3]	TOG[3]	BIO[3]	NCK[3]	CTL[3]
fa/fa	958 ± 52	849 ± 54	784 ± 35*	803 ± 32*	756 ± 34*	1059 ± 48
Lean	611 ± 26	594 ± 16	616 ± 36	613 ± 11	586 ± 30	622 ± 25

[1]Raw data for Figure 18
[2]Significant diet x genotype interaction (P=0.0073)
[3]Values are means±SEM, n=10
* Significant pre-determined contrast (P<0.05) between a treatment group and CTL

Table 19 - The effect of 9,11, 10,12, TOG, BIO, NCK and CTL diets on homeostasis model assessment index of insulin resistance (HOMA-IR) in Zucker rats [1].

HOMA-IR (units)	9,11[2]	10,12[2]	TOG[2]	BIO[2]	NCK[2]	CTL[2]	Genotype Mean[3]
fa/fa	234 ± 21	152 ± 13	122 ± 14	146 ± 10	112 ± 9	227 ± 39	165 ± 10◆
Lean	13.9 ± 2.6	11.3 ± 1.7	10.6 ± 1.3	10.6 ± 1.3	11.5 ± 1.6	11.7 ± 1.4	11.6 ± 0.7◆

[1]Raw data for Figure 19
[2] Values are means±SEM, n=10
[3] Values are means±SEM, n=60
◆ Significant genotype effect (P<0.0001)

Table 20 - The effect of 9,11, 10,12, TOG, BIO, NCK and CTL diets on homeostasis model assessment index of insulin resistance (HOMA-IR) in Zucker rats [1,2].

Diet Groups	Means (units)
9,11[3]	124 ± 27^{a}
10,12[3]	82 ± 17^{bc}
TOG[3]	66 ± 15^{c}
BIO[3]	78 ± 16^{bc}
NCK[3]	62 ± 12^{c}
CTL[3]	119 ± 31^{ab}

[1]Raw data for Figure 20
[2]Significant diet effect (P=0.0009)
[3] Values are means±SEM, n=20. Means with different letters are significantly different (P<0.05), while means with the same letter are not different (P>0.05) by Duncan's Multiple Range test.

Table 21 - The effect of 9,11, 10,12, TOG, BIO, NCK and CTL diets on serum triglyceride levels in Zucker rats [1].

Serum Triglycerides (mmol/L)	9,11[2]	10,12[2]	TOG[2]	BIO[2]	NCK[2]	CTL[2]	Genotype Mean[3]
fa/fa	4.01 ± 0.75	5.61 ± 0.90	4.18 ± 0.53	5.25 ± 0.45	5.00 ± 0.56	5.24 ± 1.09	4.88 ± 0.30◆
Lean	0.63 ± 0.05	0.59 ± 0.08	0.55 ± 0.06	0.56 ± 0.05	0.63 ± 0.07	0.49 ± 0.08	0.58 ± 0.03◆

[1]Raw data for Figure 21
[2] Values are means±SEM, n=10
[3] Values are means±SEM, n=60
◆ Significant genotype effect (P<0.0001)

Table 22 - The effect of 9,11, 10,12, TOG, BIO, NCK and CTL diets on serum free fatty acid levels in Zucker rats [1].

Serum Free Fatty Acids (mmol/L)	9,11[2]	10,12[2]	TOG[2]	BIO[2]	NCK[2]	CTL[2]	Genotype Mean[3]
fa/fa	1.27 ± 0.07	1.41 ± 0.09	1.31 ± 0.10	1.30 ± 0.11	1.15 ± 0.10	1.24 ± 0.05	1.27 ± 0.04◆
Lean	0.55 ± 0.03	0.55 ± 0.03	0.50 ± 0.05	0.52 ± 0.03	0.49 ± 0.03	0.51 ± 0.05	0.52 ± 0.02◆

[1]Raw data for Figure 22
[2]Values are means±SEM, n=10
[3]Values are means±SEM, n=60
◆ Significant genotype effect (P<0.0001)

Table 23 - The effect of 9,11, 10,12, TOG, BIO, NCK and CTL diets on skeletal muscle AMPK-α levels in Zucker rats [1,2].

AMPK-α (arbitrary units, relative to control)	9,11[3]	10,12[3]	TOG[3]	BIO[3]	NCK[3]	CTL[3]
fa/fa	1.16 ± 0.07	1.03 ± 0.06	0.86 ± 0.05	1.03 ± 0.04	1.00 ± 0.04	1.00 ± 0.06
Lean	1.13 ± 0.12	1.12 ± 0.15	1.19 ± 0.20	1.13 ± 0.17	1.13 ± 0.21	1.11 ± 0.18

[1]Raw data for Figure 23
[2]No significant main effects (P>0.05)
[3]Values are means±SEM, n=7

Table 24 - The effect of 9,11, 10,12, TOG, BIO, NCK and CTL diets on skeletal muscle phospho-AMPK-α levels in Zucker rats [1,2].

Phospho-AMPK-α (arbitrary units, relative to control)	9,11	10,12	TOG	BIO	NCK	CTL
fa/fa	1.29 ± 0.06[3]	1.37 ± 0.07[3]	1.55 ± 0.19[3]	1.67 ± 0.22[3]	1.52 ± 0.16[3]	1.33 ± 0.16[3]
Lean	1.43 ± 0.14[4]	1.60 ± 0.21[4]	1.90 ± 0.29[4]	2.03 ± 0.35[4]	2.25 ± 0.43[4]	2.07 ± 0.37[4]

[1]Raw data for Figure 24
[2]No significant main effects (P>0.05)
[3]Values are means±SEM, n=6
[4]Values are means±SEM, n=7

Table 25 - The effect of 9,11, 10,12, TOG, BIO, NCK and CTL diets on skeletal muscle phospho-AMPK-α / AMPK-α ratios in Zucker rats [1,2].

Phospho-AMPK-α / AMPK-α (ratios)	9,11[3]	10,12[3]	TOG[3]	BIO[3]	NCK[3]	CTL[3]
fa/fa	1.18 ± 0.08	1.44 ± 0.12	1.92 ± 0.24	1.66 ± 0.20	1.55 ± 0.20	1.36 ± 0.22
Lean	1.39 ± 0.16	1.60 ± 0.22	2.17 ± 0.65	2.27 ± 0.60	2.72 ± 0.74	2.75 ± 0.93

[1]Raw data for Figure 25
[2]No significant main effects (P>0.05)
[3]Values are means±SEM, n=6

Table 26 - The effect of 9,11, 10,12, TOG, BIO, NCK and CTL diets on skeletal muscle TNF-α (19 kDa) levels in Zucker rats [1].

Skeletal Muscle TNF-α (19 kDa) (arbitrary units, relative to control)	9,11[2]	10,12[2]	TOG[2]	BIO[2]	NCK[2]	CTL[2]	Genotype Mean[3]
fa/fa	0.95 ± 0.05	0.80 ± 0.09	1.03 ± 0.15	1.42 ± 0.21	1.18 ± 0.07	1.11 ± 0.12	1.08 ± 0.08◆
Lean	1.63 ± 0.26	1.78 ± 0.34	1.91 ± 0.38	1.77 ± 0.33	1.03 ± 0.11	0.76 ± 0.05	1.48 ± 0.18◆

[1]Raw data for Figure 26
[2] Values are means±SEM, n=4
[3] Values are means±SEM, n=24
◆ Significant genotype effect (P<0.0479)

Table 27 - The effect of 9,11, 10,12, TOG, BIO, NCK and CTL diets on skeletal muscle TNF-α (26 kDa) levels in Zucker rats [1].

Skeletal Muscle TNF-α (26 kDa) (arbitrary units, relative to control)	9,11[2]	10,12[2]	TOG[2]	BIO[2]	NCK[2]	CTL[2]	Genotype Mean[3]
fa/fa	0.78 ± 0.07	0.77 ± 0.06	0.97 ± 0.17	1.14 ± 0.27	0.76 ± 0.12	0.69 ± 0.14	0.85 ± 0.10◆
Lean	1.66 ± 0.34	1.64 ± 0.33	2.03 ± 0.49	1.68 ± 0.22	1.20 ± 0.20	0.84 ± 0.12	1.51 ± 0.19◆

[1]Raw data for Figure 27
[2] Values are means±SEM, n=4
[3] Values are means±SEM, n=24
◆ Significant genotype effect (P=0.0078)

Table 28 - The effect of 9,11, 10,12, TOG, BIO, NCK and CTL diets on total skeletal muscle TNF-α levels in Zucker rats [1].

Total Skeletal Muscle TNF-α (19 kDa + 26 kDa)	9,11[2]	10,12[2]	TOG[2]	BIO[2]	NCK[2]	CTL[2]	Genotype Mean[3]
fa/fa	1.72 ± 0.10	1.58 ± 0.14	2.00 ± 0.32	2.55 ± 0.47	1.94 ± 0.18	1.80 ± 0.25	1.93 ± 0.17◆
Lean	3.29 ± 0.60	3.42 ± 0.66	3.94 ± 0.80	3.45 ± 0.54	2.23 ± 0.19	1.61 ± 0.09	2.99 ± 0.35◆

[1] Raw data for Figure 28
[2] Values are means±SEM, n=4
[3] Values are means±SEM, n=24
◆ Significant genotype effect (P=0.0105)

Table 29 - The effect of 9,11, 10,12, TOG, BIO, NCK and CTL diets on skeletal muscle TNF-α ratios in Zucker rats [1].

Skeletal Muscle TNF-α Ratio (19 kDa / (19 kDa + 26 kDa))	9,11[2]	10,12[2]	TOG[2]	BIO[2]	NCK[2]	CTL[2]	Genotype Mean[3]
fa/fa	0.55 ± 0.02	0.51 ± 0.01	0.53 ± 0.01	0.58 ± 0.03	0.63 ± 0.03	0.63 ± 0.03	0.57 ± 0.02◆
Lean	0.51 ± 0.02	0.53 ± 0.02	0.52 ± 0.04	0.49 ± 0.03	0.48 ± 0.06	0.50 ± 0.06	0.50 ± 0.02◆

[1]Raw data for Figure 29
[2] Values are means±SEM, n=4
[3] Values are means±SEM, n=24
◆ Significant genotype effect (P=0.0339)