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Manipulating Lipolysis to Reduce Fatness and Improve Carcass Composition in Commercial Broilers

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I am submitting herewith a thesis written by Rodney Barnett Ray entitled "Manipulating Lipolysis to Reduce Fatness and Improve Carcass Composition in Commercial Broilers." I have examined the final electronic copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Animal Science.

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Manipulating Lipolysis to Reduce Fatness and Improve Carcass Composition in Commercial Broilers

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Dedicated to my family: My parents, Delaina and Rodney; my aunt, Angela; my grandparents, Bertha and Charles; my dog, Riley, and everyone else whose encouragement and pride in me served as a constant source of strength. Without them this would not have been possible.

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Abstract

Betaine has been studied as an osmolyte and methyl group donor for many species. Recent studies have found that betaine is able to increase muscle mass and reduce adiposity in meat type broilers birds. In experiment one, eight-hundred Cobb 500 broiler chicks were supplemented with betaine at 0.6 g/kg and 1.2g/kg. Birds had access to feed and water for ad libitum consumption for forty-two, forty-four, and forty-nine days. Birds and feed were weighed weekly. Carcass parameters (dress weight, breast percentage, and leg percentage) were taken at slaughter on days forty- two and forty-nine. Blood was taken at day forty-four to assess non-esterified fatty acids, triglycerides and glucose. Betaine did not have an effect on carcass performance, triglycerides or glucose. Betaine did however have a significant effect on NEFA concentration ($p<0.05$) indicating a betaine induced change in lipolysis.

In experiment two, ten one day old broilers were used to establish a protocol for lipolysis in broiler adipose explants. Adipose depots were harvested, cultured and treated with a variety of known lipolytic agents and nuclear receptor agonists. Media concentrations of glycerol and NEFA were measured to assess lipolytic activity of the explants. Treatments included: glucagon, isobutyl-1-methylxanthine(IBMX), isoproterenol, betaine, TO-901317, GW-7647 and rosiglitazone. Bezafibrate, IBMX, glucagon and TO-901317 had an significant effect on media glycerol and non-esterified fatty acid concentration ($p<0.05$) indicating an increase in lipolysis.

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Chapter 1 Introduction

The poultry industry is under increased pressure to produce mass quantities of meat in a fast and efficient manner. While the poultry industry is streamlined to produce meat efficiently, there are certain metabolic inefficiencies that need to be overcome to produce a more profitable animal. In the process of creating a leaner animal through diet manipulation, it is possible to find solutions to our own metabolic shortcomings. Adipose tissue metabolism is a mystery that when unraveled can provide answers and applications that range from the farm to the playground and everywhere in between. The first step in understanding this mystery is to discover a method to augment the metabolic process of the adipocyte. Lipolysis in the chicken; what works?

Betaine is a water soluble, naturally-produced organic base that has the potential to improve tolerance of heat stress by both protecting against cellular dehydration and improving lipid metabolism. In vivo, betaine is supplied by dietary sources and by the oxidation of choline. Betaine plays a dual physiological role as both an important cellular osmolyte and a methyl donor for the re-methylation of homocysteine to methionine.

Betaine's osmotic importance stems from its accumulation within cells, where it participates in the control of cell volume. As an osmolyte, betaine may have a stabilizing function on cells subjected to osmotic disorders such as that which may occur in coccidiosis, conditions where ionophores are used. Dehydration of cells occurs during infections and use of betaine may have the potential to improve these conditions.

Metabolically, betaine affects lipid partitioning in the body. Betaine supplementation reduces lipid deposition in liver, most clearly evidenced in its ability to reverse fatty liver in

models of nonalcoholic fatty liver disease (Wang, Yao et al. 2010). Betaine has also been shown to reduce deposition of abdominal adipose tissue in several animal models, including geese and broiler chickens. Improved adiponectin levels, improved plasma insulin, and improved glucose levels have been associated with betaine supplementation; implying that betaine has the potential to improve adipose tissue function and insulin sensitivity (Wang, Yao et al. 2010). Greater fat deposition in broilers selected for high body weight or high abdominal fat content is associated with increased plasma concentrations of insulin and glucagon (Sinsigalli, McMurtry et al. 1987; Dupont, Chen et al. 1999).

The second function of betaine is as that of a methyl donor. Betaine supplies methyl groups for the re-methylation of homocysteine to methionine, a reaction catalyzed by betaine homocysteine methyl transferase (BHMT). The dual functions of betaine as an osmolyte and as a methyl donor are not mutually exclusive because BHMT is osmoregulated, with high tonicity reducing its expression so that betaine metabolism decreases when osmolyte concentrations need to be maintained (Schäfer et al. 2007). The conversion of homocysteine to methionine is important to conserve methionine, detoxify homocysteine, and produce S-adenosylmethionine (SAM)(Barak et al. 1996). S-adnenosylmethionine's importance in fat metabolism is observed in its role in phosphatidylethanolamine methyltransferase (PEMT) activity (Hoffman et al. 1981). The PEMT pathway synthesizes phosphatidylcholine, and is a vital step in the synthesis and secretion of very low density lipoproteins (Noga et al. 2002).

Collectively, betaine supplementation may have dual benefit in conditions of heat stress in poultry by both improving lipid metabolism and attenuating cellular dehydration. Beyond heat stress, its impact on adipose physiology could benefit the broiler industry by reducing carcass fatness and thus improving feed efficiency.

In addition, to betaine, there are other viable options to augment adipose metabolism in chicken. In mouse models both hormonal and chemical treatments have stimulated lipolysis in adipocytes. The major metabolic hormones glucagon and insulin have been shown to increase and decrease lipolysis respectively. Chemical agents that include forskolin, isoproterenol and 3-isobutyl-1methylxanthine (IBMX) have also demonstrated the ability to up regulate the lipolytic pathway in various ways including the following: activation of adenylyl cyclase, up regulation of cyclic AMP, and acting as an agonist to the β-adrenergic receptors.

Chapter 2 Literature Review

Avian Metabolism

Carbohydrate metabolism in poultry is characterized by hyperglycemia and insulin resistance (Vasilatos-Younken 1986). The glucose transporter type 4 (GLUT4), which is the insulin responsive transporter in mamamls, is absent in some birds such as chicken and sparrow (Seki et al. 2006; Sweazea and Braun 2006) but present in others such as ducks (Thomas-Delloye et al. 1999). GLUT4 is responsible for insulin-regulated glucose transport into the cell.

In the fed state, glucose levels in adult poultry range from 190 to 220 mg/dl (Hazelwood 1986); juvenile plasma glucose levels range from 156 mg/dl to 330 mg/dl (Scanes 2008). Some studies report that fasting does not affect circulating glucose concentrations (Belo et al. 1976; Hazelwood and Lorenz 1959; Tinker et al. 1986) while others report the contrary (Harvey et al. 1978). However, there are small consistent decreases in circulating concentrations of glucose following twenty-four hour feed withdrawal in meat type poultry (Edwards et al. 1999) .

It is evident that glucagon plays a role in maintaining glucose homeostasis in poultry. Fasting results in an increase in plasma glucagon (Edwards et al. 1999). Glucagon is the major lipolytic hormone in chickens (Campbell and Scanes 1987) and has been shown to reduce feed intake in chicks (Honda et al. 2007). Insulin at high concentrations can depress glucagon-stimulated lipolysis with chicken adipose tissue in vitro(Langslow and Hales 1969). Despite the presence of circulating insulin at the expected levels, chickens present a status similar to mammalian type 2 diabetic status possessing hyperglycemia and low sensitivity to exogenous insulin (Akiba et al. 1999).

The critical enzyme in gluconeogenesis is Phosphoenolpyruvate carboxykinase (PEPCK), especially in the kidney. Adult female chicks lack PEPCK activity in the kidney while juvenile birds possess PEPCK (Tinker et al. 1983). The enzyme retains its activity in the liver, but the reduced activity of the enzyme over time indicates that gluconeogenesis efficiency decreases throughout the bird's lifetime.

Betaine

Betaine, the common name for trimethylglycine, is also known as glycine betaine, lycine and oxyneurine (Craig 2012). Betaine was first discovered in the juice of the sugar beet (*Beta vulgaris*) in the 19th century. It was then isolated in other organisms soon after. Betaine rich foods, in order of concentration, include the following: wheat bran (1339 mg/100g), wheat germ (1241 mg/100g), spinach (600-645 mg/100g), beets (114-297 mg/100g), pretzels (237 mg/100g), shrimp (219mg/100g), wheat bread (201mg/100g), and crackers (49-199mg/100g). Betaine's function is to serve as an organic osmolyte to protect cells under stress or as a methyl donor, which provides methyl groups for various biochemical pathways.

Betaine absorption occurs in the duodenum with serum concentration peaking at one to two hours after ingestion (Craig 2012). Betaine is catabolized primarily in the liver and kidney, and in these tissues a series of transmethylation reactions occur with the most important transferring a methyl group from betaine to homocysteine (Craig 2012). In this series of reactions betaine is converted to dimethylglycine, and homocysteine becomes methionine (Craig 2012). The remethylation of homocysteine is catalyzed by Betaine-homocysteine methyltransferase (BHMT) (Li, Feng et al. 2008).

As stated, one of betaine's most important functions is that of a methyl donor. Therefore, to understand Betaine's role as a methyl donor its relationship with other methyl donors must also be understood. Methionine and Choline are of particular interest because of the interplay between the three compounds. All three are commonly added to poultry diets and betaine, a metabolite of choline, has a slight sparing effect on choline (Dilger et al. 2007).

Choline

Historically choline, along with manganese, folic acid, nicotinic acid, and biotin, has been added to feed to prevent perosis (slipped tendon), but it has also been proven that it is needed in larger amounts than other vitamins because choline deficiency will result in decreased growth, and poor feed utilization (Nesheim et al. 1979). Good sources of choline include: fish meal, yeast, liver meal, and soybean oil meal (North 1984). Many phospholipids, including phosphatidylcholine, lysophosphatidylcholine, sphyngomyelin, lysosphyngomyelin and plasmalogens, contain a choline constituent (Simon 1999). This is of importance because phospholipids contribute to the integrity of the cell membrane (Simon 1999).

Choline most likely plays a role in fat metabolism because choline deficiency is associated with the development of fatty liver due to a reduction in lipoprotein assembly (Simon 1999). Chicks may at times synthesize choline but at insufficient levels, therefore choline must be added to starter rations (North 1984). Choline synthesis is a function of both age and sex. Older birds synthesize more than younger birds, and hens produce more choline than roosters (Nesheim et al. 1979; North 1984).

One Carbon Metabolism

The major pathway associated with the exchange of methyl groups and the methyl donors betaine, choline and methionine is the one catalyzed by BHMT. Choline is oxidized to betaine by the enzyme choline oxidase.(Kidd et al. 1997). Hydrolysis of betaine generates one free methyl group and a molecule of dimethylglycine. The free methyl group is transferred to homocysteine by the action of BHMT and the dimethylglycine is further degraded to sarcosine and glycine (Simon 1999). When methionine is deficient and there is an abundance of choline and betaine; BHMT activity is increased (Emmert et al. 1996).

Conversion of choline to betaine is an inefficient process because choline must be transported from the cytosol into the mitochondria before it can be oxidized to betaine (Kidd et al. 1997). Betaine is then transported across the mitochondrial membrane into the cytosol where it can function as methyl group donor (Mann et al. 1938). This makes choline conversion especially difficult in chickens. Polyether ionophores are commonly used to control coccidiosis in poultry, and these ionophores are known to interfere with mitochondrial membrane transport of choline (Tyler 1977).

Betaine Hydroxy-methyltransferase

BHMT deficiency results in a general improvement in metabolic state by improving glucose tolerance and insulin sensitivity in mice (Teng et al. 2012). In addition to the metabolic improvement associated with BHMT deficiency, mice deficient in BHMT also possessed smaller fat pads, with inguinal and gonadal fat up to 41% smaller than fat pads in wild type mice (Teng et al. 2012). The small size of the fat depots can be attributed to smaller adipocyte size

(approximately 80% smaller) in BHMT deficient mice (Teng et al. 2012). These data along with the increase in adipocyte number associated with deficient mice implies that adipocyte differentiation was not affected by the deficiency but rather adipocyte storage and utilization of triglyceride (Teng et al. 2012) . Overall energy usage is also altered by BHMT deficiency. Mice genetically deficient in BHMT consumed the same amount of feed ,and showed no difference in lipid absorption and excretion compared to wild type mice, but demonstrated increased O_2 consumption and CO_2 excretion throughout light and dark phases which is an indication of increased energy usage (Teng et al. 2012). BHMT null mice possessed a basal insulin concentration that was about 50% less than control mice which indicates greater insulin sensitivity, while other plasma measures including glycerol, non-esterified fatty acids, and glucose showed no difference between genotypes (Teng et al. 2012). The BHMT deficient mice also expressed a hyper metabolic phenotype which could be attributed to a 33% increase in plasma thyroxine (T_4) (Teng et al. 2012). BHMT knockout mice and wild type mice had similar lipolytic rates (Teng et al. 2012). This is a curious finding because the knockout mice possess smaller fat pads and increased energy expenditure. However, when treated with isoproterenol, wild type and knockout mice demonstrated similar levels of triacylglycerol and glycerol, while knockout mice demonstrated a 50% increase in plasma glucose concentration. The observed glycerol concentration similarity was consistent with explant trials (Teng et al. 2012).

Betaine is currently used in the poultry industry to protect chicks from coccidiosis because it can alleviate symptoms and improve performance (Fetterer et al. 2003). Betaine has also been shown to cause a slight decrease in abdominal fat in broiler birds, when added to the finishing diet (Xing et al. 2011). This decrease in adiposity may be attributed to betaine's effect on fibroblast growth factor (FGF21) expression. FGF21 is a recently discovered FGF and is

thought to regulate metabolism (Nishimura et al. 2000). Betaine hydroxyl-methyltransferase deficiency results in an accumulation of betaine in tissues, and causes and increase in FGF21 expression. This increase in FGF21 expression is thought to be due to increased energy expenditure in mouse models (Teng et al. 2012). Betaine and its role in FGF21 expression are ideal targets for lipolysis in the chicken because energy status is the key regulator for adipose tissue metabolism (Ji et al. 2012).

Betaine is obtained naturally by both consumption of foods with high betaine and by the conversion of choline to betaine. In fact betaine has a slight sparing effect on choline; betaine can replace up to 50% of the dietary choline requirement (Dilger et al. 2007). Exposure to specific stressors such as drought, high salinity, or temperature stress can trigger the synthesis of betaine in mitochondria which subsequently increases betaine concentration within cells (Craig 2012). Osmoregulation is defined as the ability of a cell to maintain its structure and function by regulating water movement in and out of the cell (Kidd et al. 1997). When betaine is not catabolized, i.e. when cellular concentration increases within cells it is used as an organic osmolyte. This function allows betaine to regulate the hydration state of a cell as well as maintain cellular function (Craig 2012). Some metabolic pathways sensitive to a change in hydration include protein turnover, amino acid metabolism, ammonia metabolism, carbohydrate metabolism, fatty acid metabolism, plasma membrane transport, bile excretion, pH control and gene expression (Häussinger 1996). Organic osmolytes such as betaine are favorable because they do not have the detrimental effects of inorganic osmolytes. Inorganic intracellular osmolytes include potassium, magnesium and phosphate. These osmolytes have a limited function because high concentrations of inorganic osmolytes, caused by osmotic stress, can affect metabolic function (Yancey PH 1982). High concentrations of sodium chloride and

potassium chloride can alter the kinetics of enzymes within a cell but enzyme kinetics are not affected in cells with high concentrations of organic osmolytes such as betaine and choline. Therefore, in periods of prolonged hyperosmotic stress, cells will rely on the accumulation of organic osmolytes to attenuate the effects of stress while avoiding the detrimental effects of inorganic osmolytes (Burg 1994). Betaine is ideal as an additive to poultry feed because it is one of the major organic osmolytes found to increase in the renal cells of the avian along with myo-inositol, and taurine (Lien et al. 1993).

Ex Vivo Studies

In vitro experiments are ideal for focused metabolic experimentation because of the ease with which pathways can be directly by pharmacological or nutrient treatments. In vitro studies require minimal live animals, if any, for sample collection and the small scale of the experiments require less treatment material without sacrificing power. A number of in vitro studies have been done using adipose tissue explants and mature adipocytes. These experiments provide rationale for ambitious in vivo trials.

Betaine

In humans, betaine supplementation alleviated insulin resistance in vitro (Kathirvel et al. 2010). Human HepG2 cells were made insulin resistant by exposure to high (30mM glucose) for 24 hours and induced with 10 nM insulin for ten minutes. The activation of protein kinase B in these insulin resistant cells was reduced by 50% when compared to control models. Betaine supplementation restored protein kinase B activation by fifty to one hundred percent depending

on betaine concentration (Kathirvel et al. 2010) The cells grown in high glucose media also possessed higher levels of phosphorylated 5'AMP- activated protein kinase (AMPK) (Kathirvel et al. 2010). AMPK acts as a metabolic regulator and monitors energy stasis by sensing the AMP: ATP ratio of the cell. AMPK phosphorylation suppresses fatty acid synthesis by inhibiting acetyl- CoA carboxylase, the rate limiting enzyme in the fatty acid synthesis pathway (Kathirvel et al. 2010). When there is high ATP in a cell the kinase is active signaling for the storage of fat. When insulin resistant HepG2 cells were treated with betaine at varying concentrations AMPK phosphorylation was suppressed. This suggests that betaine might reduce hepatic fat synthesis (Kathirvel et al. 2010)

The effect of BHMT on adiposity has been demonstrated in vitro. BHMT deficiency leads to impaired triacylglycerol synthesis and enhanced glucose oxidation in isolated mature adipocytes, as well as impaired triacylglycerol oxidation in liver homogenates. Although fat explants from both BHMT-deficient and wild type mice demonstrated the same rate of triglyceride synthesis, BHMT-deficient mice synthesized sixty-two percent less triacylglycerol (Teng et al. 2012).

Conjugated Linoleic acid

Conjugated linoleic acids (CLA) are a group of isomers of conjugated octadecadienoic acid that occur naturally and are found in milk and in the meat of ruminants (Terpstra 2004). The isomers which exhibit biological activity are CLA trans-10, cis-12(t-10,c-12) and CLA cis-9 , trans-11(c-9,t-11)(Vyas et al. 2012). Conjugated linoleic acid has been acknowledged to have numerous biological activities. Metabolically it has been shown to reduce lipid uptake in adipocytes leading to changes in whole body composition (Oku et al. 2003; Park et al. 1997;

Park et al. 1999). Active isomers of CLA appear to reduce lipid in two ways. First, conjugated linoleic acids inhibit fatty acid synthase, which limits fatty acid biosynthesis (Furlan et al. 2013). Second, CLA reduces the activity of perilipin1, a coat protein of lipid droplets that plays a role in lipolysis and lipogenesis of adipose tissues, which causes a reduction in white adipose tissue and aberrant lipolysis in mouse tissue in vitro (Cai et al. 2012). In rats CLA can reduce epididymal and retroperitoneal adipose depots by 26% and 53% respectively (Furlan et al. 2013). When supplemented at 1.5% CLA diminished adipose tissue at a rate that caused difficulty in harvesting adipose samples (Cai et al. 2012).

In humans, the effect of CLA on body composition and metabolism needs more investigation. There is evidence that CLA can lower body fat mass in both obese and healthy individuals (Blankson et al. 2000; Thom et al. 2001). However, it is unclear whether this reduction in fat mass is due to exercise or the increased CLA intake.

While the benefit of CLA supplementation in humans is unclear, there are documented cases of CLA's usefulness in the agricultural sector. In cattle, average daily gain increased significantly in animals supplemented with CLA at 1.72 kg/day in the form of linseed. However, average daily intake and carcass classification (conformation and fat cover) were not affected by CLA supplementation (Albertí et al. 2013). Similar findings have been reported in goats with no effect on dry matter intake, body weight, milk yield, milk protein content, and lactose yield. However, CLA has been shown to decrease milk fat yield, a function of de novo fatty acid synthesis (Ghazal et al. 2012).

In poultry, CLA is used not as a metabolic regulator or carcass enhancer, but as an immunity booster. Infectious bursal disease virus (IBDV) is a significant disease in the poultry

industry because of its effect on the immune system and its virulence (Long et al. 2011). Because CLA has been shown to alleviate immunosuppression it is the perfect weapon to combat IBDV (Long et al. 2011). In chickens infected with IBDV, CLA significantly reduced mRNA expression of inflammatory cytokines, interferon gamma and interleukin 6, as well as attenuated the pathological changes in the bursa caused by IBDV (Long et al. 2011).

Although conjugated linoleic acid has a number of benefits it must also be noted that there are significant side-effects to CLA supplementation. The trans-10, cis-12 isomer has been shown to cause severe hyperinsulinemia and insulin resistance in mice, hamsters and pigs (Bouthegourd et al. 2002; Clement et al. 2002; Roche et al. 2002; Stangl et al. 1999; Tsuboyama-Kasaoka et al. 2000).

Lipolysis

The adipocyte is the primary site of storage of fat for use as energy in times of negative energy balance. Free-fatty acids from the diet and from de novo fatty acid synthesis are transported to the adipocyte and esterified to glycerol, a product of glucose metabolism, to form a triglyceride molecule. These triglycerides are stored within the lipid droplet of the adipocyte. In vertebrates, triacylglycerol-rich lipid droplets of adipocytes provide a major energy storage depot for the body (Brasaemle 2007).

The ability to store energy in the form of triglycerides and the ability to use these energy stores in times of low carbohydrate availability, or when metabolic demand is high, is a highly conserved process across species and is essential for survival (Lampidonis et al. 2011). Obesity occurs when energy intake exceeds energy need and there is a pathologic accumulation of white adipose tissue (Zechner et al. 2005). Adipose tissue homeostasis is regulated by a number of

neural and hormonal signals, but the complexity of these pathways can be understood best if viewed as the "metabolic equilibrium" between triglyceride synthesis and storage (Zechner et al. 2005). Adipose tissue lipolysis is the process of breaking down stored triglyceride within the adipocyte resulting in the release of non- esterified fatty acid (NEFA) and glycerol (Lampidonis et al. 2011).

It is widely accepted that a cyclic AMP-dependent mechanism is the rate-limiting step in lipolysis in fat cells. The initial step in the process involves the hormonal activation of adenylate cyclase which results in an increase in the production of cyclic AMP (cAMP) and increased activation of cyclic AMP-dependent protein kinase (protein kinase A; PKA) and the subsequent phosphorylation and activation of hormone sensitive lipase (Belfrage et al. 1982). Cyclic AMP can be increased hormonally by glucagon or chemically by isoproterenol or forskolin (Allen et al. 1986; Ravnskjaer et al. 2013).

In most species hormone sensitive lipase is one of the major targets of this regulation. Binding of agonists to the β-adrenergic receptors, coupled to adenylate cyclase leads to activation of protein kinase A which in turn phosphorylates HSL and stimulates lipolysis (Holm 2003). In vitro, phosphorylation of hormone sensitive lipase by PKA can be monitored as an increased activity against triglycerides and cholesteryl ester substrates. In vivo, phosphorylation via PK A results in the translocation of HSL from a cytosolic location, to the surface of the lipid droplet (Clifford et al. 1997; Egan et al. 1992).

In addition, to HSL, perilipin proteins are major targets for PKA phosphorylation upon β-adrenergic stimulation of adipocytes. Perilipins are a family of closely related proteins that are abundantly expressed on the surface of adipocytes (Londos et al. 1999). The role of perilipin in

PKA-mediated lipolysis is apparent in perilipin null mice. These mice demonstrated= attenuated β-adrenergic-induced lipolysis (Holm 2003). Perilipin appears to play a dual role as suppressor of basal lipolysis and as a necessary component for full lipolytic stimulation to occur (Souza et al. 1998). It has also been demonstrated that without fully functioning perilipin A, translocation of hormone sensitive lipase to the lipid droplet cannot occur (Sztalryd et al. 2003).

Adipocyte lipolysis is regulated hormonally and HSL is one of the major targets of this regulation (hence its name). Insulin is believed to be the most important anti-lipolytic hormone in mammals. In most species, insulin inhibits lipolysis in adipocytes through inhibition of hormone sensistive lipase (Anthonsen et al. 1998). This is because of insulin's ability to lower cAMP levels and thus PKA activity. Insulin accomplishes this through activation of phosphodiesterase 3B (Shakur et al. 2001). The anti-lipolytic activity of insulin can be countered with the treatment of a phosphodiesterase inhibitor such as 3-isobutyl-1-methylxanthine (IBMX). Phosphodiesterases degrade cAMP and control its levels within the cell. Persistent elevation of cAMP due to treatment with IBMX prevents insulin-mediated suppression of lipolysis (Makino et al. 1992).

There are also a number of nuclear hormone receptors that could affect lipolysis. Peroxisome proliferator activated receptors (PPARs) are the most important of these receptors, in particular PPARγ. Activated PPARγ increases the expression of key adipogenic genes that promote the storage of fatty acids and decreases the expression of lipolytic genes (Ferre 2004). The formation of preadipocytes from mesenchymal stem cells, as well as differentiation of preadipocytes, is dependent on the activation of PPARγ (Rosen et al. 1999)

Another nuclear receptor associated with lipid metabolism is the liver X receptor alpha (LXRα). There is a relationship between inflammation and lipid metabolism and the LXR family of receptors integrate metabolic and inflammatory signaling (Bensinger et al. 2008). The liver X receptors (LXRα and LXRβ) control intracellular sterol and lipid homeostasis by regulating the genes involved in cholesterol transport, disposal, lipogenesis and glucose metabolism.(Laurencikiene and Ryden 2012) Oxysterols, oxidized derivatives or cholesterols, serve as ligands to activate LXRs. (Archer et al. 2013; Laurencikiene and Ryden 2012). The role of LXRα is well documented within the liver but it is not well understood how LXRα influences lipid metabolism in white adipose tissue (WAT). Both LXRα and LXRβ are expressed in WAT (Steffensen et al. 2003). In mice, activation of LXRα with a synthetic ligand resulted in increased lipogenesis and increased storage of lipid in sub-cutaneous adipose depots (Archer et al. 2013) However, activation of LXRα by use of agonists have been shown to increase lipolytic rates in other species (Cruz-Garcia et al. 2012).

Previous work in our lab has found that the release and oxidation side of the balance equation are also important determinants of fatness in chickens. There is evidence that as leanness increases, circulating non-esterified fatty acids and the rate of fatty acid oxidation also increase. Genetically lean lines of chickens have higher levels of lipolysis and increased fatty acid oxidation in adipose tissue (Ji et al. 2012). These studies serve as the basis for the current investigation of the efficacy of lipolysis as a method for carcass modification in broilers.

Chapter 3 Materials and Methods

In Vivo Supplementation

Experimental Rationale

Betaine has been shown to reduce adiposity and increase breast weight in broilers in the absence of methionine (Zhan et al. 2006). However it is not clear how betaine affects the whole bird in a diet with normal methionine. It is also important to note that Zhan and others did not study betaine in the absence of other methyl group donators(Zhan et al. 2006). Therefore, it is important to test the effects of betaine when added to a normal poultry ration. Because betaine appears to be a suitable replacement for methionine as far as growth is concerned, it is assumed that when combined, with methionine, betaine will result in an increase in lean growth and decrease in adiposity.

Experimental Design

Eight hundred, one day-old mixed sex broiler chicks (Cobb 500), obtained from Pilgrims Corporation (Cohutta,Georgia), were weighed in groups of 10, wing-banded, and assigned to floor pens across two rooms. Each room represented different growth environments. Eight replicated pens were assigned to each of five dietary treatments. Mash diets were prepared from a corn-soybean meal based common diets. Phase feeding was carried out in which a starter diet (3038 kcal/kg,, 21% protein) was fed from day 1 to day 21, a grower diet (3077 kcal/kg, 20% protein) from day 22 to day 38 and a finisher diet (3124 kcal/kg, 18% protein) from day 39 to termination of the study. The basal diet for all treatments was formulated to meet NRC requirements, with some nutrients at greater levels to match levels in typical commercial diets; however, methionine and choline were set to NRC levels. Dietary treatments were as follows:

1. Control - basal diet with choline level formulated to NRC level
2. Choline 1 – basal diet supplemented with choline chloride to provide 500 ppm of methyl equivalent.
3. Choline 2 - basal diet supplemented with choline chloride to provide 1000 ppm of methyl equivalent.
4. Betaine 1- basal diet supplemented with betaine to provide 500 ppm of methyl equivalent.
5. Betaine 2 - basal diet supplemented with betaine to provide 1000 ppm of methyl equivalent.

Feed and water were provided ad libitum throughout the experiment. Chicks were grown in a 23 hour light: 1 hour dark cycle with incandescent light supplementation. Birds were group weighed once weekly. Any birds that died during the experiment were weighed and the date of death and the weight of the bird were recorded and added to the group weight for that week.

Starting on day 22, one of the temperature-controlled rooms was maintained at 23.9° C (thermo-neutral) while the temperature in the other room was cycled between 23.9° C and 33 °C (heat stress) in eight hour intervals. Body weight was monitored weekly and used to determine weight gain. Blood was collected at weeks four and five from birds that were slaughtered at weeks six and seven, respectively, and again at day 44 from birds that were not slaughtered. Blood was allowed to clot for at least 30 minutes and then centrifuged to separate serum, which was collected and stored at -20^{o}C until use for assays. The resultant serum was used to determine the effect of treatment on lipid metabolism.

Measures of non-esterified fatty acids (NEFA), triglyceride content, glucose and glycerol were perfromed using commercial assay kits. Non esterified fatty acids were analyzed using HR Series NEFA-HR(2) (Wako Chemicals USA, Richmond, Virginia). The NEFA assay relies on

the acylation of coenzyme A (CoA)by the fatty acids in the presence of added acyl- CoA synthetase (ACS). The acyl-CoA is oxidized by added acyl- CoA oxidase with the generation of hydrogen peroxide. Hydrogen peroxide, with peroxidase permits the condensation of 3-methyl-N-ethyl-N-(β-hydroxyethyl)-aniline (MEHA) with 4-aminoantipyrine to form a purple colored product which can be measure colorimetrically at 550 nm. The levels of NEFA represent the amount of fatty acids that are not bound to a glycerol backbone and indicate the level of lipolysis occurring in the animal. NEFA is presented in milligrams per deciliter (mg/dl)

Triglyceride content was analyzed using L-Type TG M (Wako Chemicals USA, Richmond Virginia). Serum triglycerides indicate the level of lipid available to the body via the circulation. The triglyceride assay works similarly to the NEFA assay but relies on the decomposition of glycerol to glycerol-3-phosphate. Glycerol is converted to glycerol-3-phosphate by glycerol kinase in the presence of adenosine-5'-triphosphate (ATP). Glycerol-3-phosphate formed is oxidized by glycerol-3-phosphate oxidase in a reaction that produces hydrogen peroxide. The hydrogen peroxide produced causes HMMPS and 4-aminoantipyrine to undergo a oxidative condensation catalyzed by peroxidase (POD), producing a blue pigment. Glucose was measured using the Glucose Colorimetric Assay Kit (Cayman Chemical, Boston, Massachusetts). The glucose assay uses the glucose oxidase peroxide reaction for the determination of clucose concentration. In this reaction glucose is oxidized to δ-gluconolactone with simultaneous reduction of glucose oxidase.. the reduced glucose oxidase is regenerated to its oxidized form by molecular oxygen to produce hydrogen peroxide. Then horseradish peroxidase catalyzed a reaction between hydrogen peroxide and 3,5-dichloro-2-hydroxybenzenesulfonic acid and 4-aminoantipyrine to generate a pink color that can be measured coloremetrically at 514 nm and expressed as (mg/dl) Glucose utilization represents the

overall metabolic state of the animal. Glycerol content was measured using the free glycerol reagent (Sigma-Aldrich, St. Louis, Missouri). The Free Glycerol Reagent measures free, endogenous glycerol using coupled enzyme reactions. Glycerol is phosphorylated by adenosine-5-triphosphate (ATP) forming glycerol-1-phosphate (G-1-P) and adenosine-5-diphosphate (ADP) in the reaction catalyzed by glycerol kinase (GK). G-1-P is then oxidized by glycerol phosphate oxidase (GPO) to dihydroxyacetone phosphate (DAP) and hydrogen peroxide. Peroxidase (POD) catalyzes the coupling of hydrogen peroxide with 4-aminoantipyrine (4-AAP) and sodium N-ethyl-N-(3-sulfopropyl) m-anisidine (ESPA) to produce a purple dye that can be measured at 540 nm and is expressed in milligrams per milliliter (mg/ml) Serum gycerol concentration serves as an additional measure of lipolysis, in conjunction with NEFA measurement. Glycerol and NEFA should have a positive relationship, with an expected ratio of three molecules of NEFA for each molecule of glycerol.

On day 42 and again on day 49, following a 12-hour fast, three birds from each pen were randomly selected, slaughtered and processed into market parts. The carcass was divided into leg quarter and breast portions and part yield was calculated as percent of the whole carcass. On day 44, sixty birds of the same sex were euthanized and blood, abdominal fat and liver were collected, weighed, and preserved for downstream analyses.

Statistical Analysis:

The experiment was a completely randomized split-plot design, with temperature in the whole plot and treatment applied to pen sub-plot experimental units. Response variables were treated as repeated measures and added to the model; all analyses used mixed model analysis of

variance (SAS 9.2, Cary, NC). Least squares means were compared using Fisher's protected LSD and differences considered significant at the 5 % level of probability. Data were examined for normality using the Shapiro-wilk test for normality and equal variance . Correlation analyses were used to study relationships among the response variables.

In Vitro Stimulation of Lipolysis

Experimental Rationale

Whole body physiology is complex and there is still much to be learned about the chicken in general. With that in mind it is intuitive that the lipolytic pathway should be understood on a cellular or tissue level to make in vivo manipulation of lipolysis more efficient. Lipolysis is regulated by a cAMP dependent mechanism (Belfrage et al. 1982). Therefore, up regulation of cAMP either through increased production via adenylate cyclase or inhibition of phosphodiesterase degradation of cAMP should increase the rate of lipolysis within the adipocyte. For that reason, treatments were chosen that would have either a direct or indirect influence on cAMP concentration.

Before treatment could begin the appropriate experimental material had to be identified. We chose to develop a model using adipose tissue explants, which are small pieces of intact adipose tissue maintained in tissue culture media ex vivo. Explant cultures, unlike isolated primary adipocytes or preadipocytes, maintain the cell-cell communication among multiple cell types that is present in adipose tissue. . To maintain the same level of glycemia in which the tissue functioned in vivo, the glucose concentration of the tissue culture media was altered to approximate the glycemic status of a few chicken.

Experimental Design

Ten one day-old broiler chicks were obtained from Hubbard Cooperation (Pikeville, TN). At seven days of age, chicks were weighed and wing-banded. Commercial starter chick ration was used. Feed and water were provided ad libitum throughout the experiment. Chicks were grown in a 23 hour light:1 hour dark cycle with incandescent light supplementation. Chicks were euthanized with carbon dioxide and adipose tissue was harvested and cultured for experiments, as described below.

Collection and Processing of Adipose Tissue Explants for Tissue Culture

Materials

Adipose Tissue Collection

1. Sterile scalpel and scissors
2. Sterile 50 ml tubes
3. 37°C water bath
4. Warm transport medium (M199(Sigma-Aldrich, St. Louis, Missouri)with penicillin and streptomycin or PBS)
5. 70% ethanol
6. Chlorohexidine
7. Sterile 4x4 gauze
8. Gloves
9. Two 1 liter beakers

Explant Culture

1. Sterile tubes
2. Sterile scissors and forceps
3. Tissue culture incubator set at 37°C 95% O_2 and 5% CO_2
4. 37°C water bath
5. M199 media supplemented with Pennicillin/streptomycin and glucose at a concentration of 15mM
6. Amphotericin B
7. 100mm tissue culture dish

8. 100mm petri dish
9. Laminar flow hood

Methods

Adipose Tissue Collection

1. Warm 50 ml of transport medium in water bath. Once media is warm, weigh the tube.
2. Euthanize the chicken.
3. Wash the carcass and remove any debris.
4. Pluck the feathers around the dissection area. The fat pad is located between the keel (breast bone) and the vent (cloaca) of the bird. Thoroughly remove all feathers from the area. It is ideal to remove the feathers from the legs as well.
5. Soak sterile 4x4 gauze pads in beakers, one containing 70% ethanol and one containing chlorohexidine.
6. Clean the plucked area with chlorohexidine first. Allow approximately 2 minutes of contact. Next wash the area using the 70% ethanol soaked 4x4. Change gloves after completing this step.
7. Make an incision and remove the skin until adipose tissue is visible. Resect as much tissue as possible without contaminating tissue with gut contents.

Steps 8-9 optional

8. Wash tissue in 70% ethanol for 10 seconds followed by a PBS wash
9. Place tissue in warmed transport media and weigh. Use this weight and the weight of the tube without fat to calculate adipose tissue yield.

Explant Culture

1. Calculate the number of dishes based on weight of the tissue harvested. Dishes should not exceed (500mg/100mm dish). This proportion of tissue to media can be scaled down to accommodate smaller plates if needed. Examples: (150-250mg/60-mm dish or 50-100mg/35-mm dishes).

The following steps take place in laminar flow hood as sterile conditions are required.

2. Calculate and prepare culture media (15ml-media/ 100mm dish). Warm the media in 37° C water bath

   ***optional* add antimycotic. Amphotericin B at a concentration of 25ug/ml works well.**

3. Pour transport media and harvested tissue into petri dish. (Don't overfill the petri dish. This dish is just for tissue processing)
4. Mince the tissue into approximately 20mg pieces.
5. Distribute the tissue uniformly to 100mm tissue culture plates containing 15ml of media.
6. Incubate in cell culture incubator 37°C under 5% CO_2/95% O_2.
7. Change the media 24 hours after plating and again 48 hours after plating. This allows the tissue to recover from the collection process.
8. Calculate and prepare treatment media using the recovery media as a base.
9. Incubate and harvest accordingly.

Treatment of Adipose Explants

Explants were harvested and cultured using the protocol explained previously. After the 48 hour rest, explants were treated with the following:

Table 1. Treatments and concentrations for in vitro stimulation of lipolysis

Treatments	Concentration	Function	Solvent	Source
betaine	2mM and 20mM	Osomoprotectant, methyl donation	water	Sigma-Aldrich,
IBMX (isobutyl-1 methylxanthine)	500nM and 100nM	Phosphodiesterase inhibitor	water	Sigma-Aldrich
glucagon	100nM and 500nM	Major lipolytic hormone	50mM acetic acid	Sigma-Aldrich
isoproterenol	10μM and 1μM	B-adrenergic receptor agonist	water	Sigma-Aldrich
TO-901317	1μM and 10μM	Liver X receptor agonist	DMSO	Sigma-Aldrich,
GW-7647	1μ and 10mM	PPAR alpha agonist	DMSO	Cayman Chemical
bezafibrate	1μM and 10mM	PPAR delta agonist	DMSO	Cayman Chemical
rosiglitazone	1μM and 10μM	PPAR gamma agonist	DMSO	Sigma-Aldrich,

The treatments, concentrations and functions of various agents used to stimulate lipolysis in broiler adipose explants. All treatments contained glucose at a concentration of 15mM.

The treatments were grouped into two categories, nuclear receptor agonists and lipolytic agents. The nuclear receptors experiments included T0-901317, GW-7647, bezafibrate and rosiglitazone. The lipolytic agent experiments included betaine, IBMX, glucagon, forskolin and isoproterenol. Experiments performed were as follows:

Table 2. In vitro experiments

Experiment	Treatments	Time Points
Lipolytic Agent 1	isoproterenol, glucagon, forskolin, IBMX	1 hour, 6 hours
Lipolytic Agent 2	betaine, IBMX, glucagon	1 hour, 6 hours
Lipolytic Agent 3	betaine, IBMX, glucagon	1 hour , 6hours
Nuclear Receptor 1	rosiglitazone, TO-901317	1 hour, 24 hours
Nuclear Receptor 2	rosiglitazone, TO-901317,bezafibrate, GW-7647	1 hour, 6 hours
Nuclear Receptor 3	rosiglitazone, TO-901317, bezafibrate, GW-7647	24 hours

A list of each experiment performed, the treatments used and the time points at which media or fat was collected and stored for analysis.

Media was collected at each time point and explants were weighed and either preserved in RNAlater (Qiagen.com) or snap frozen in liquid nitrogen for RNA isolation at a later date. Non-esterified fatty acids and glycerol were measured using commercial kits. Non esterified fatty acids were analyzed using HR Series NEFA-HR(2) (Wako Chemicals USA, Richmond, Virginia) and glycerol content was measured using the free glycerol reagent (Sigma-Aldrich, St. Louis, Missouri).

Chapter 4 Results

In Vivo Supplementation of Betaine

The whole carcass weight, (weight after de-feathering and evisceration) is presented in Table 3 as dress weight. There was no significant difference in dress weight between treatments ($p>0.90$). Figure 1 represents the effects of interaction between treatment and temperature on dress weight. There was no significant effect of temperature and treatment interaction on dress weight ($p>0.60$).

The carcass was divided into market parts of leg quarter and breast. The data are presented in Table 4 and Table 5, respectively. While there was not a treatment effect on leg weight ($p>0.70$) or breast weight ($p>0.50$) there was a trend associated with temperature effect on leg weight ($p< 0.10$). Abdominal fat pad weight is presented in Table 7. There was not a significant effect of treatment ($p>0.30$) or temperature ($p> 0.80$) on fat pad weight.

The weights of the market parts were also analyzed as a percentage of dress weight. The data are presented in the appropriate Table. While the effect of treatment on breast and leg percentage was not significant, there was a trend associated with the effect of temperature on leg and breast percentage ($p<0.10$). Total muscle percentage is presented in Table 5. There was no appreciable difference across treatments ($p>0.20$) or temperature ($p>0.30$).

Figures 1-4 represent the interaction of temperature and treatment on the weight of leg quarters, breasts and abdominal fat. There was no significant difference or trend observed in the effect of temperature on carcass parameters.

Table 3. Whole carcass weight for experiment 1

Treatment	Dress weight	SEM
1	2210.69[a]	37.35
2	2223.65[a]	38.35
3	2213.73[a]	38.35
4	2247.67[a]	37.10
5	2218.86[a]	37.91
Temperature		
High Temperature	2217.82[a]	23.99
Thermoneutral	2228.02[a]	23.84

Data are least square means
[a]Values within same column not sharing letters are different ($p<0.05$)

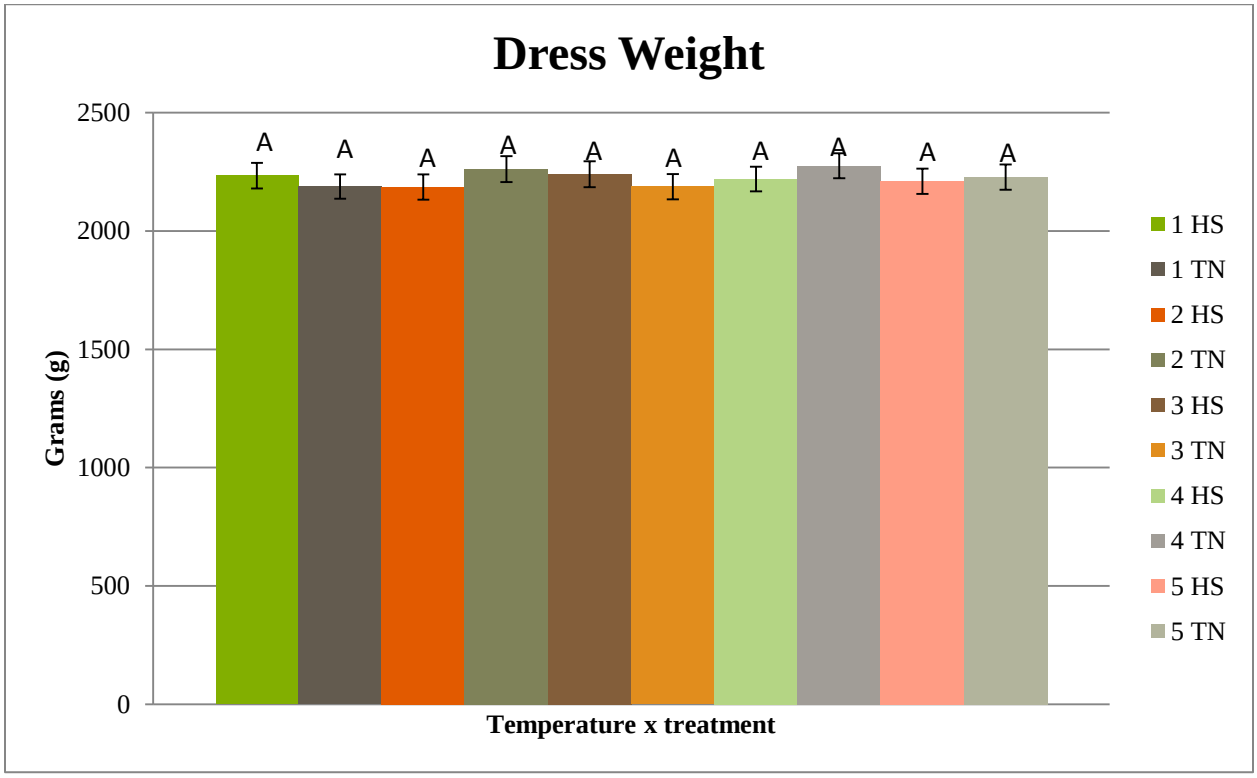

Figure 1 Interaction between treatment and temperature on dress weight. Treatments are as follows: 1-control diet, 2-choline 500ppm, 3-choline 1000ppm, 4-betaine 500ppm, 5- betaine 1000ppm Data are least square means. Values not sharing letters are different ($p<0.05$)

Table 4. Leg weight and leg percentage of total carcass for experiment 1

Treatment	leg weight	SEM	leg % of carcass	SEM
1	577.12[a]	10.21	26.1%[a]	0.00434
2	578.65[a]	10.38	26.1%[a]	0.00449
3	573.38[a]	10.32	26.1%[a]	0.00449
4	590.96[a]	10.32	26.9%[a]	0.00434
5	585.83[a]	10.32	26.1%[a]	0.00441
Temperature				
High Temperature	589.42_a	10.21	26.6%[a]	0.00280
Thermoneutral	572.96_a	10.38	25.9%[a]	0.00278

Data are least square means
[a]Values within same column not sharing letters are different ($p<0.05$)

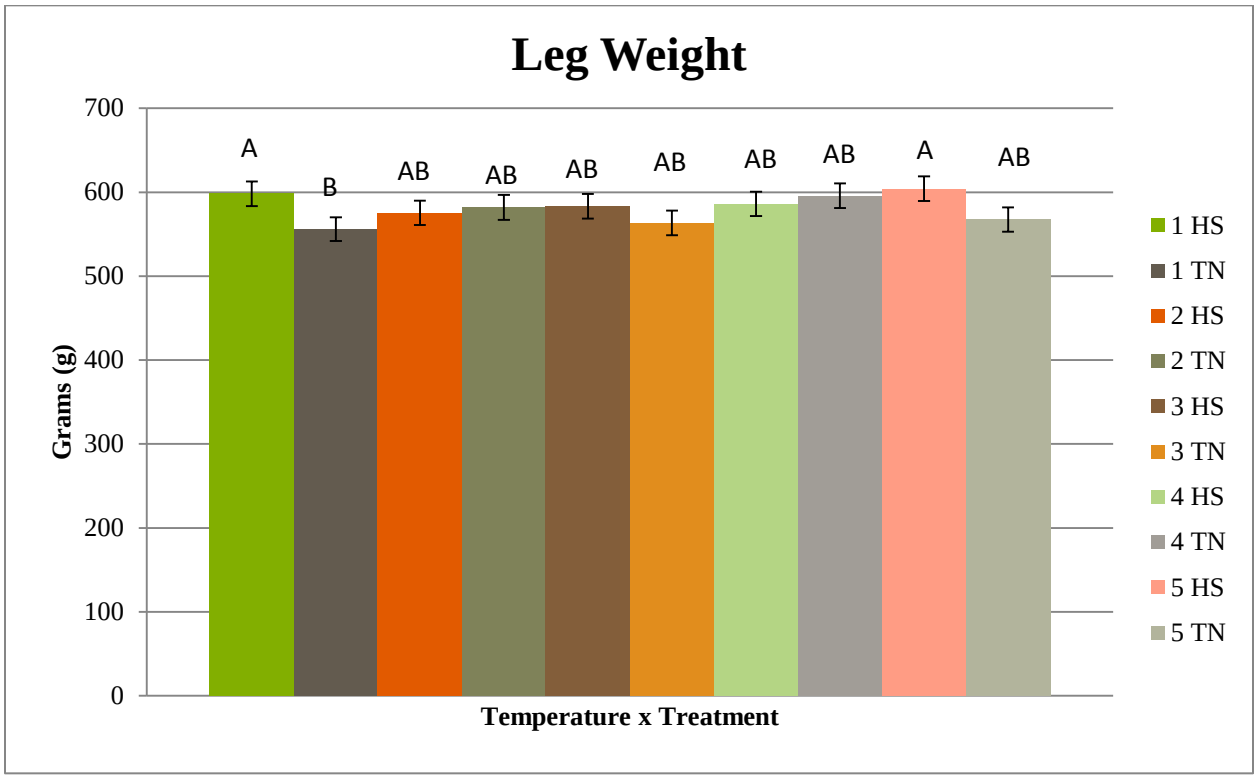

Figure 2. Interaction between treatment and temperature for leg weight. Treatments are as follows: 1-control diet, 2-choline 500ppm, 3-choline 1000ppm, 4-betaine 500ppm, 5- betaine 1000ppm Data are least square means. Values not sharing letters are different ($p<0.05$)

Table 5. Breast weight and breast percentage of total carcass for experiment 1

Treatment	breast weight	SEM	breast % of carcass	SEM
1	861.14[a]	16.734	38.9%[ab]	0.00611
2	869.51[a]	16.910	38.8%[ab]	0.00631
3	853.85[a]	16.905	38.7%[b]	0.00631
4	889.4[a]	16.818	40.4%[a]	0.00607
5	858.75[a]	16.905	39.2%[ab]	0.00620
Temperature				
High Temperature	854.52[a]	10.681	38.7%[a]	0.00394
Thermoneutral	878.54[a]	10.661	39.7%[a]	0.00390

Data are least square means
[a]Values within same column not sharing letters are different ($p<0.05$)

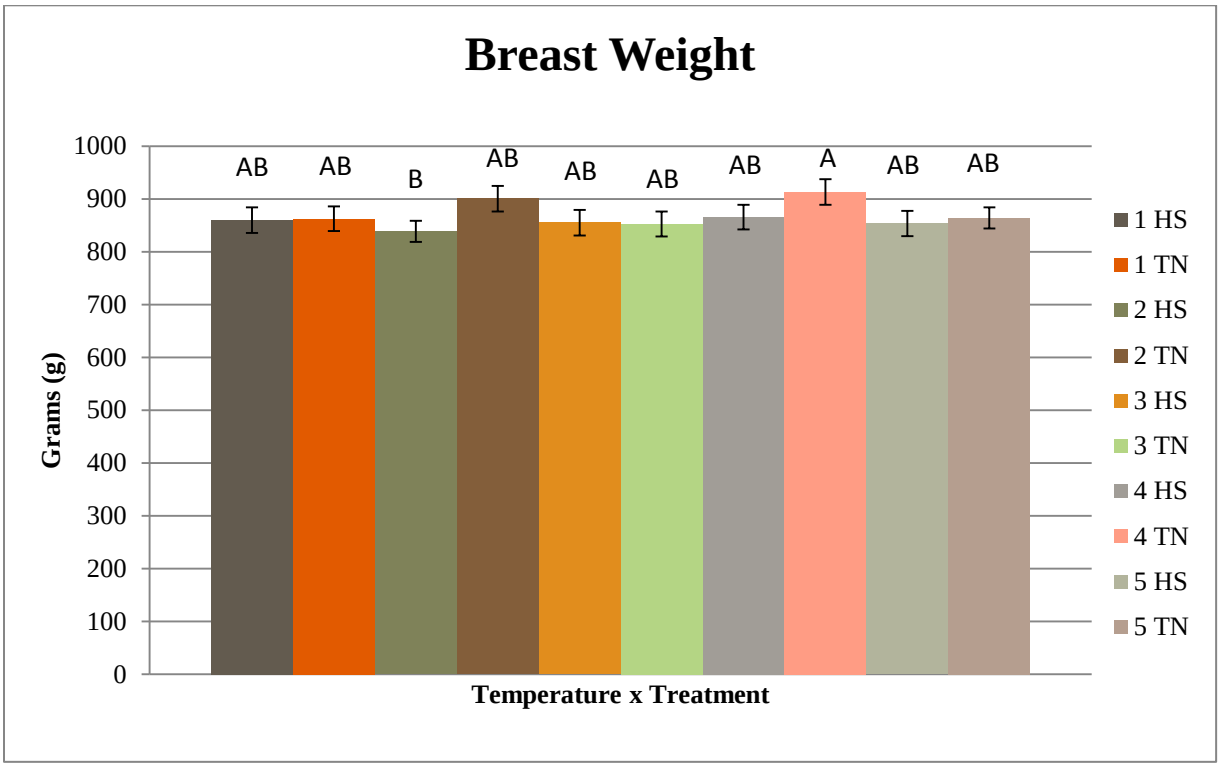

Figure 3. Temperature and treatment interaction for breast weight. Treatments are as follows: 1-control diet 2-choline 500ppm, 3-choline 1000ppm, 4-betaine 500ppm, 5- betaine 1000ppm Data are least square means. Values not sharing letters are different ($p<0.05$)

Table 6. Percentage of muscle as a function of carcass weight

Treatment	Muscle % of carcass	SEM
1	65.08%[ab]	0.01003
2	64.93%[ab]	0.01036
3	64.78%[ab]	0.01036
4	67.44%[a]	0.01003
5	64.19%[b]	0.01019
Temperature		
High Temperature	64.88%[a]	0.00648
Thermoneutral	65.68%[a]	0.00642

Data are least square means

[a]Values within same column not sharing letters are different ($p<0.05$)

Table 7. Abdominal Fat pad weight and percentage of total carcass

Treatment	Abdominal fat	Ab fat % of carcass
1	35.9393[ab]	1.7%[ab]
2	38.1879[a]	1.8%[a]
3	33.4999[b]	1.5%[ab]
4	35.7442[ab]	1.6%[ab]
5	35.611[ab]	1.6%[ab]
Temperature		
High Temperature	35.6534[a]	1.6%[a]
Thermoneutral	35.9395[a]	1.6%[a]

Data are least square means
[a]Values within same column not sharing letters are different ($p<0.05$)

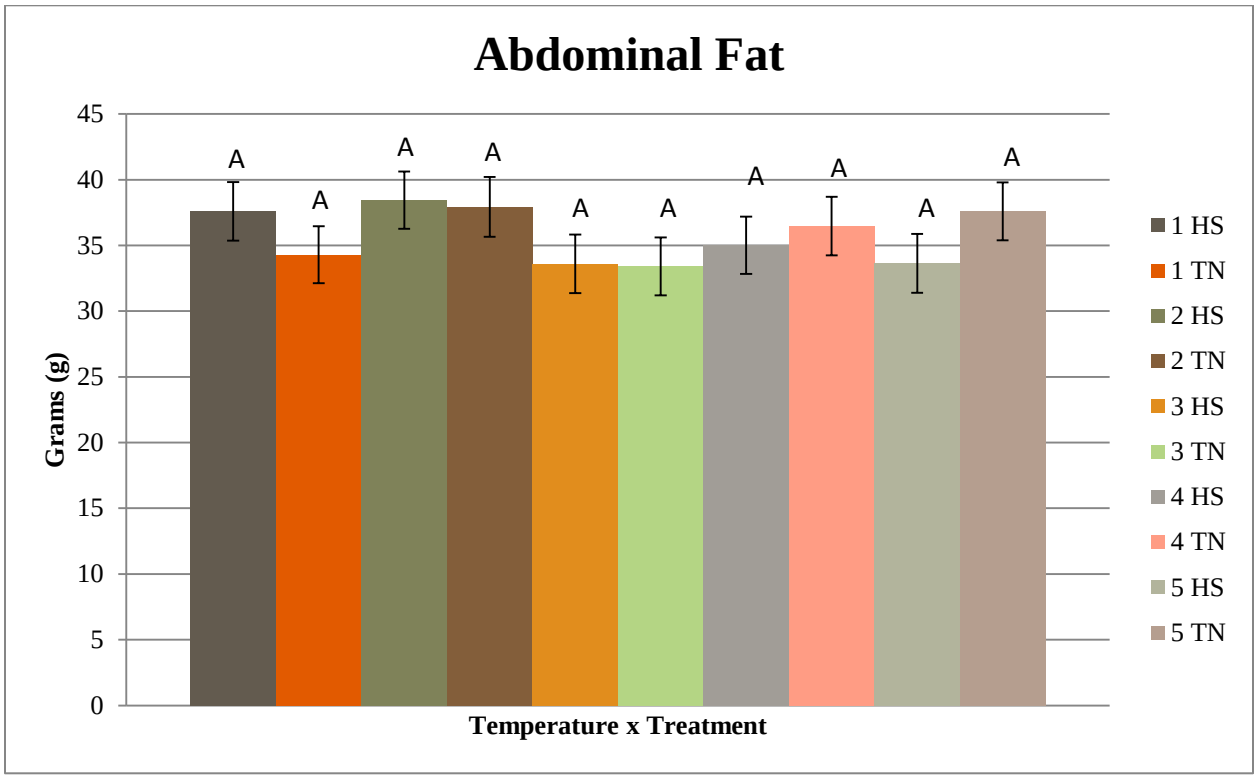

Figure 4. Temperature and treatment interaction for abdominal fat. Treatments are as follows: 1-control diet, 2-choline 500ppm, 3-choline 1000ppm, 4-betaine 500ppm, 5- betaine 1000ppm Data are least square means. Columns not sharing letters are different ($p<0.05$).

Glucose, NEFA, and triglyceride concentrations were measured in the serum of chickens slaughtered at 44 days of age. Figure 5 shows the concentration of NEFA in 44 day-old broilers fed with the two betaine diets and the control diet. Supplementation of betaine significantly altered NEFA in chickens ($p < 0.05$). Betaine did not, however, have an effect on triglyceride concentration ($p > 0.16$) .

As expected, supplementation of betaine did not have an effect on glucose concentrations ($p > 0.20$), indicating that betaine did not disrupt the normal metabolic state.

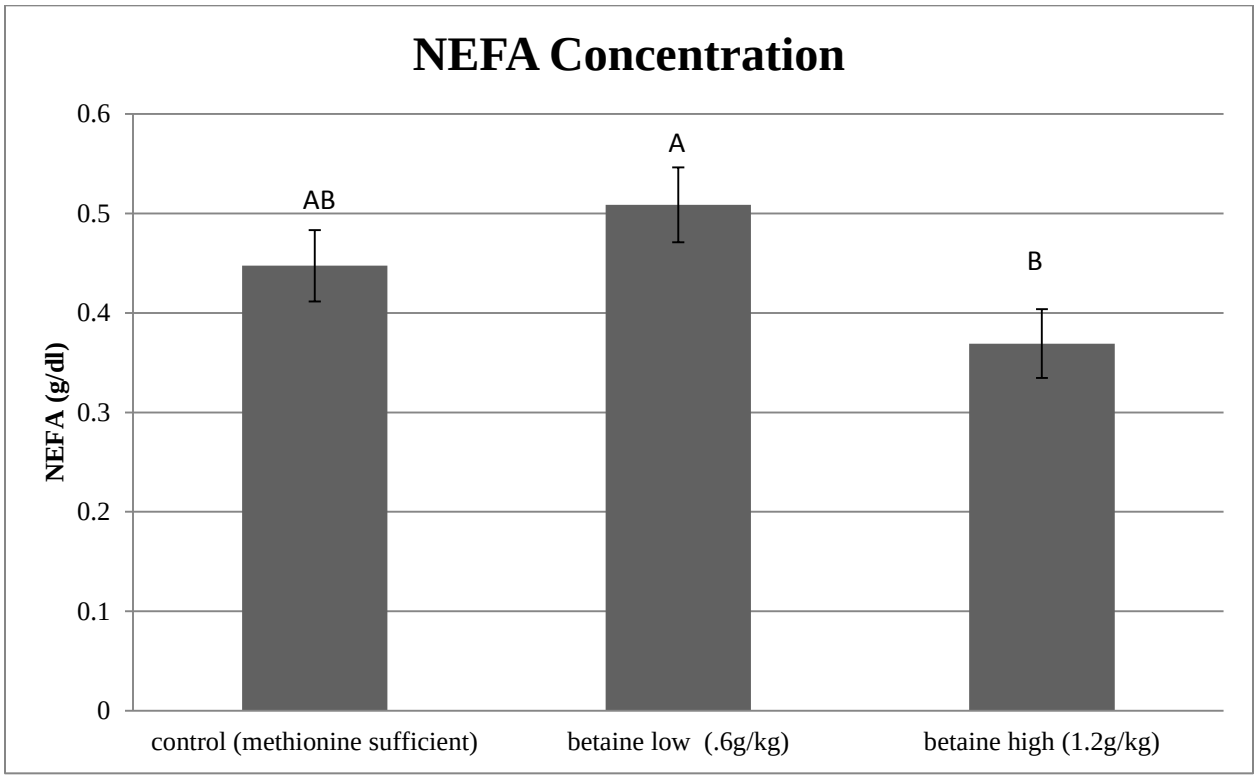

Figure 5. Serum NEFA concentration of 44 day old broilers treated with betaine. Data are least square means and columns not sharing letters are different ($p<0.05$)

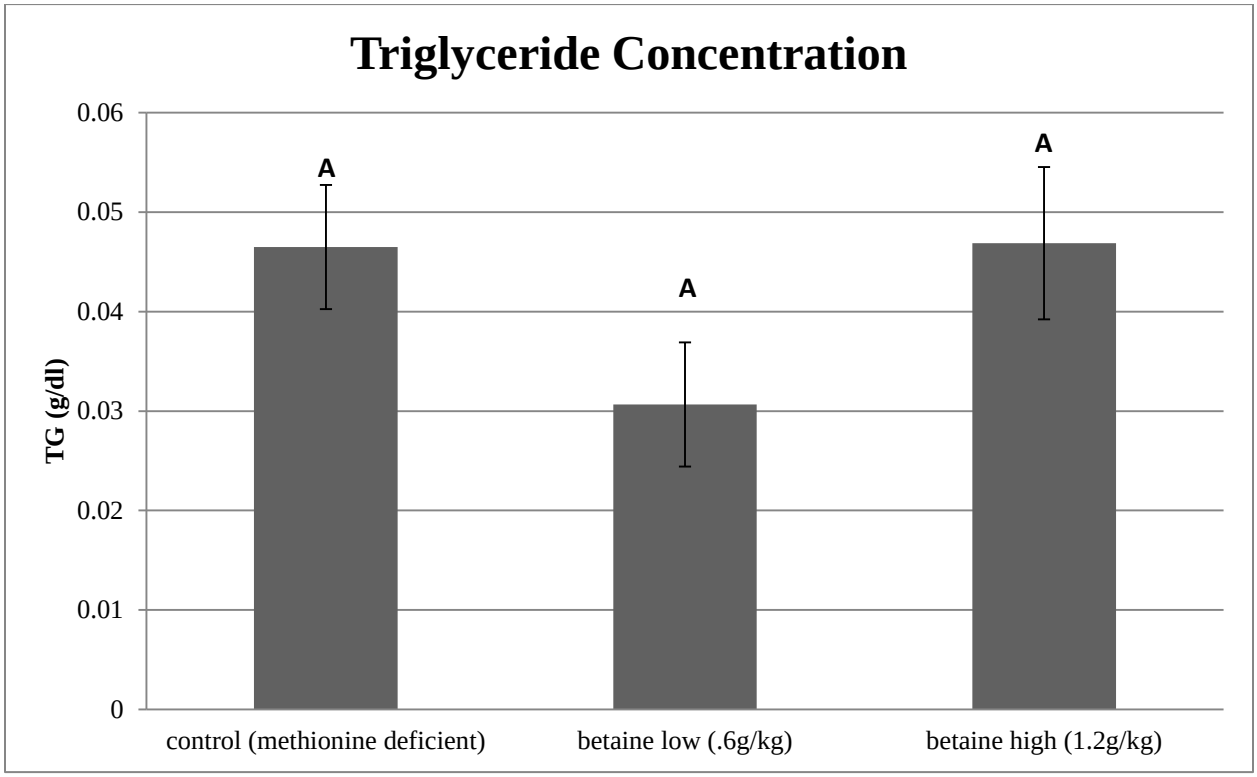

Figure 6. Serum triglyceride concentration of 44 day old broilers treated with betaine. Data are least square means and columns not sharing letters are different ($p<0.05$)

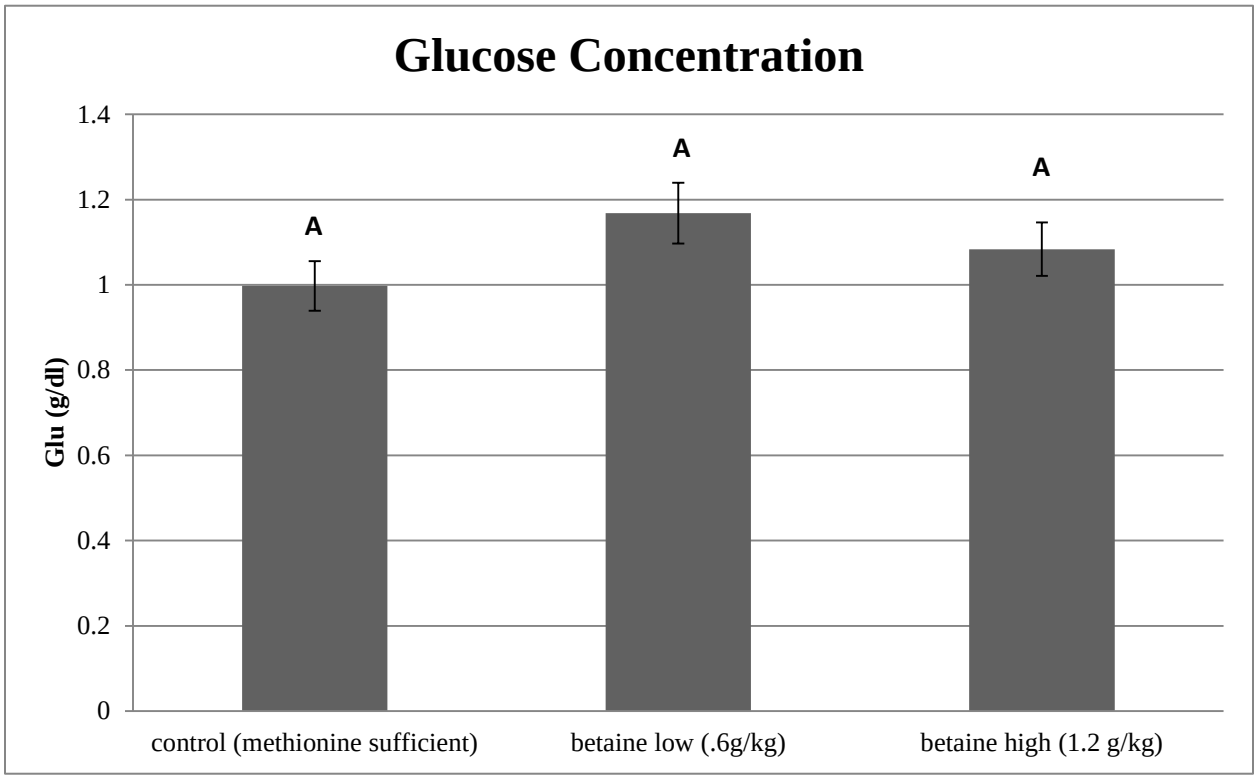

Figure 7. Serum glucose concentration of 44 day old broilers treated with betaine. Data are least square means and columns not sharing letters are different ($p<0.05$)

Ex Vivo Stimulation of Lipolysis

Media concentrations of glycerol and NEFA were used to determine the effects of various treatments on lipolysis in chicken adipose explants. One hour treatment with Isobutyl-1-methylxanthine and glucagon significantly increased media glycerol concentration ($p<0.05$;Figure 8). Both treatments also significantly increased NEFA concentration (Figure 10). The effect of IBMX on glycerol persisted after six hours ($p<0.05$; Figure 9). NEFA levels after six hours of glucagon treatment were significantly lower than controls (Figure 11). Isoproterenol treatment did not stimulate lipolysis, based on NEFA and glycerol concentrations (Figures 8-11). Betaine caused a significant increase in glycerol and NEFA concentrations after one hour of treatment ($p<0.05$; Figure 12 and Figure 14, respectively). The effects of betaine were not apparent after 24 hours of treatment (Figure 13and 15).

The addition of BSA led to increased non-esterified fatty acids in media. This is due to albumin acting as a "NEFA trap" in the media. Albumin binds to free fatty acids as a means of transportation (Spector 1975). Because of this a source of albumin either from bovine, human or mouse should be added when measuring NEFA concentration of media. The effects of BSA on media glycerol and NEFA concentrations are shown in Figures 16-19. As shown in Figures 16 and 17, there was no consistent effect of BSA supplementation on glycerol release. However, the addition of BSA significantly increase the media NEFA concentration, even in the absence of stimulation, as shown in the controls (Figure 18 and 19). In each treatment, BSA significantly increased the effect of treatment on NEFA concentration. When BSA was present in the media, betaine significantly increase NEFA release after both one and six hours of treatment ($p<0.05$, Figure 18 and 19, respectively). The effect of IBMX is inconsistent at times but this could be

due to its tendency to fall out of solution when frozen and difficulty in confirming visually that it was completely solubilized upon thawing.

Bezafibrate and TO-901317 significantly increased glycerol within the media following a six hour incubation ($p<0.05$, Figure 22). When compared to their vehicle specific control TO-901317 and bezafibrate caused a 3.6 and 1.8 fold increase in glycerol respectively. This indicates that the Liver X receptor alpha and Peroxisome Proliferator Receptor Activator delta are active and stimulating lipolysis within the adipocyte.

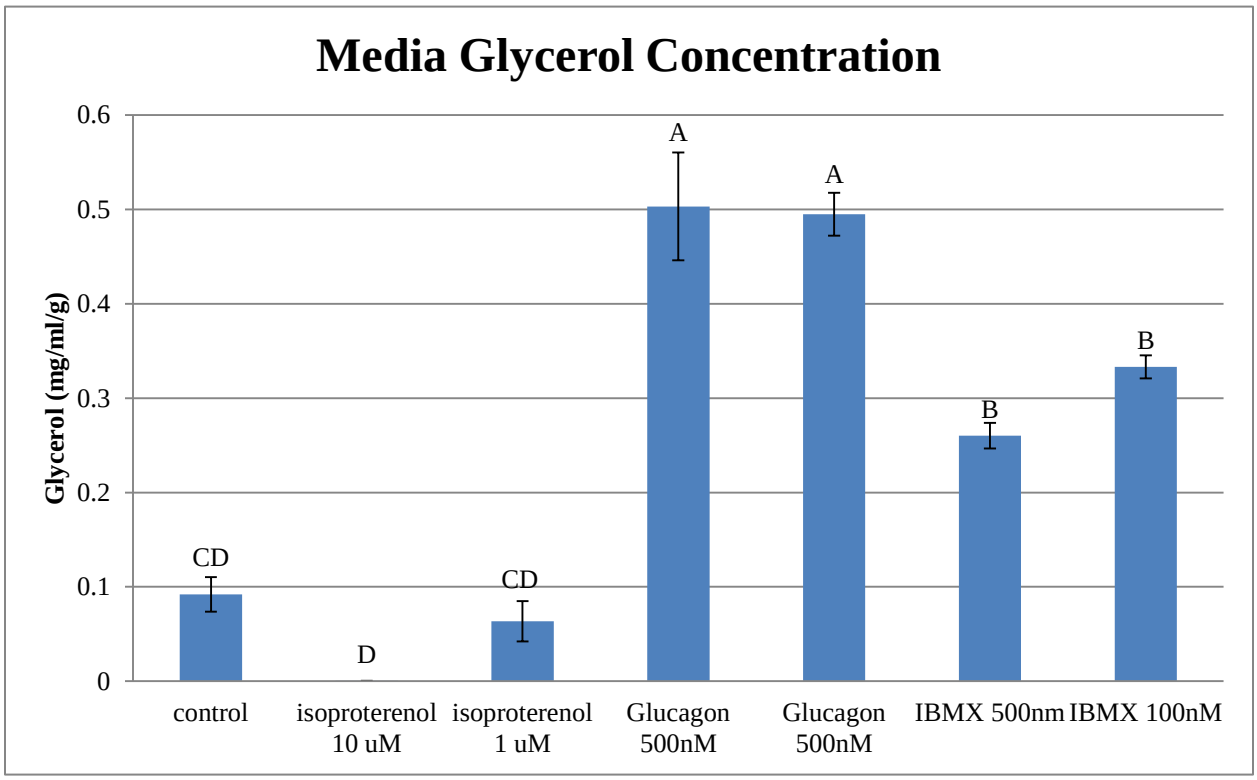

Figure 8. Effects of one hour treatment with lipolytic agents on media glycerol concentration normalized for tissue weight per well (mg glycerol/ ml media/ g tissue). Glycerol is represented as milligram per milliliter per gram of tissue. Treatment details can be found in Table 1 IBMX, Isobutyl-1-methylxanthine. Data are least square means and columns not sharing letters are different ($p<0.05$)

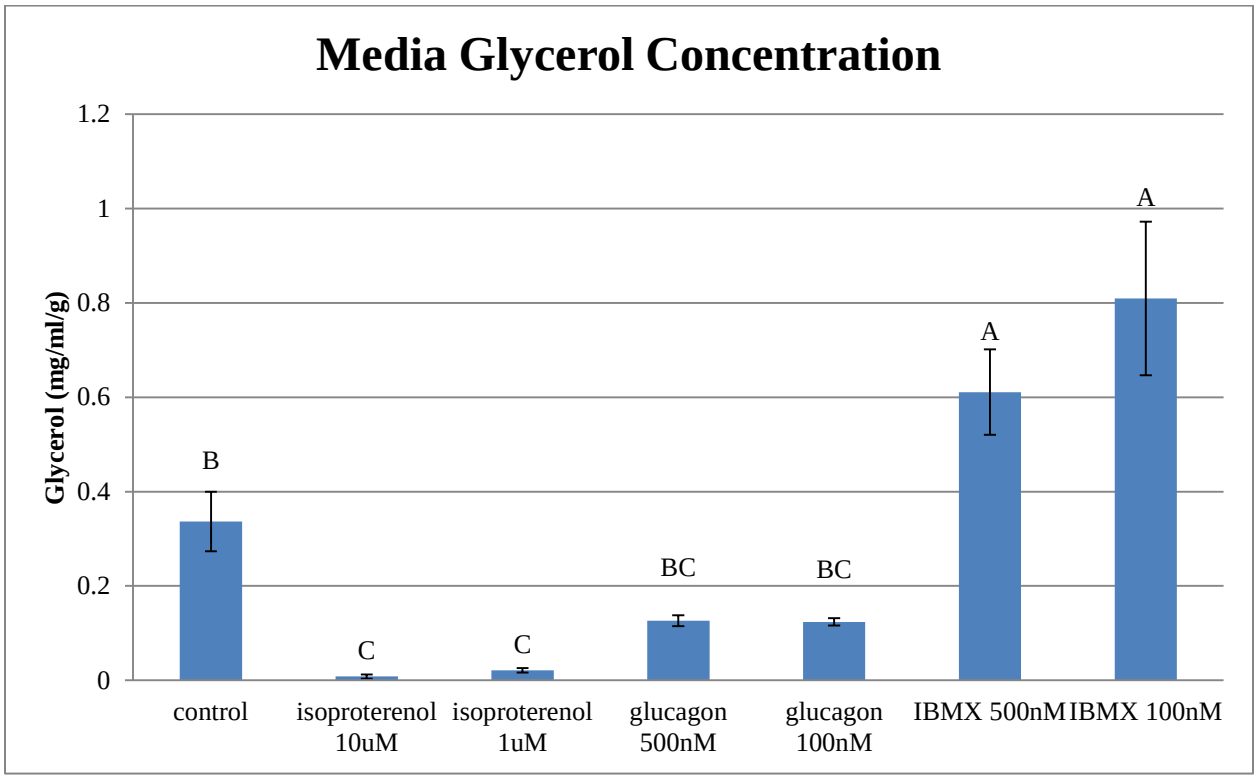

Figure 9. Effects of six hour treatment with lipolytic agents on media glycerol concentration normalized for tissue weight per well (mg glycerol/ ml media/ g tissue). Treatment details can be found in Table 1. IBMX; isobutyl-1-methylxanthine Data are least square means and columns not sharing letters are different ($p<0.05$)

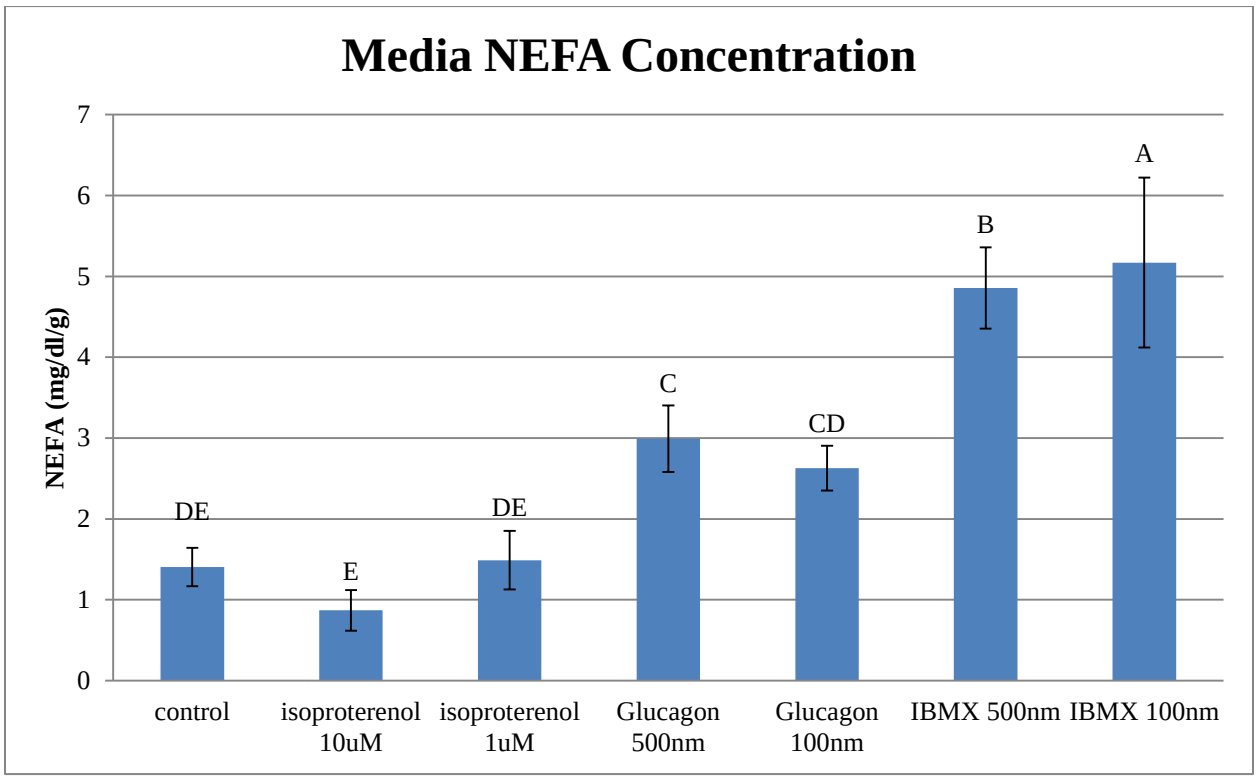

Figure 10. Effects of one hour treatment with lipolytic agents on media NEFA. Normalized for tissue weight(mg NEFA/dl media/ g tissue) NEFA is presented as grams per deciliter per gram of tissue. Treatment details can be found in Table 1. IBMX; isobutyl-1-methylxanthine. Data are least square means and columns not sharing letters are different ($p<0.05$)

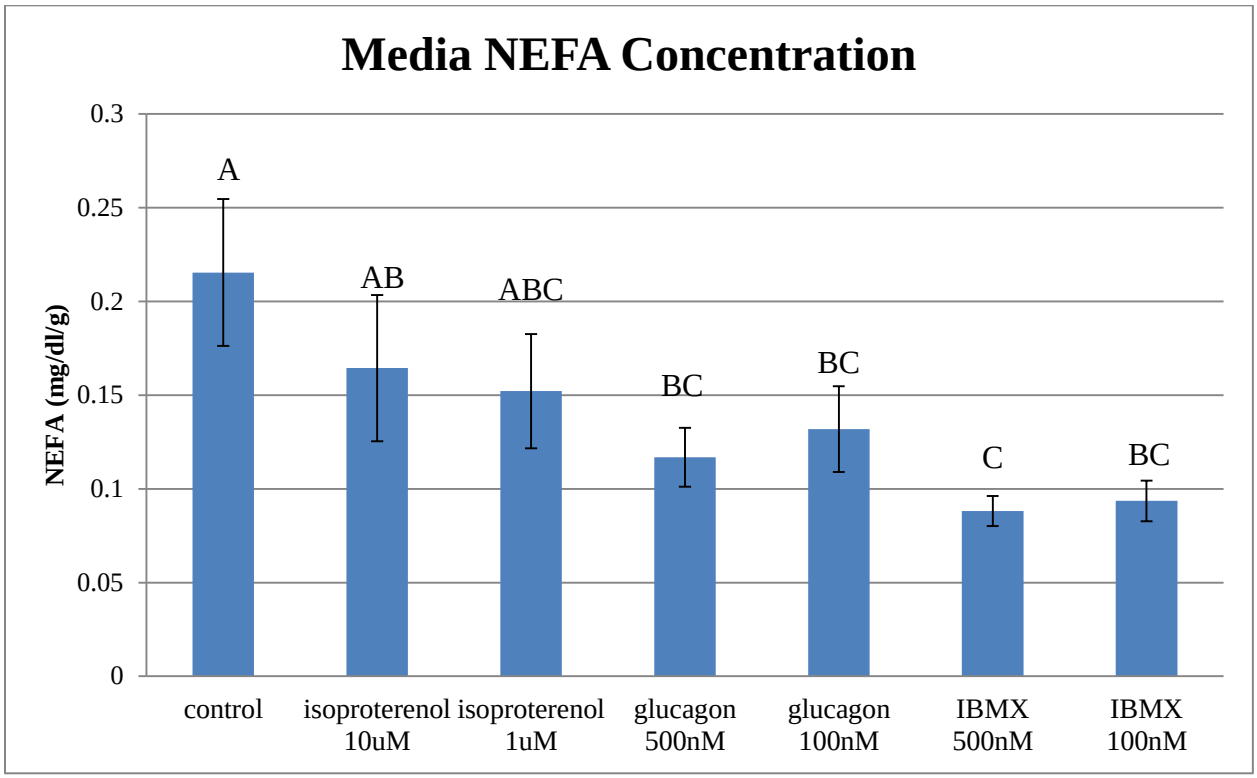

Figure 11 Effects of six hour treatment with lipolytic agents on media NEFA concentration normalized for tissue weight per well (mg glycerol/dl media/g tissue). Treatment details can be found in Table 1. IBMX; isobutyl-1-methylxanthine Data are least square means and columns not sharing letters are different ($p<0.05$)

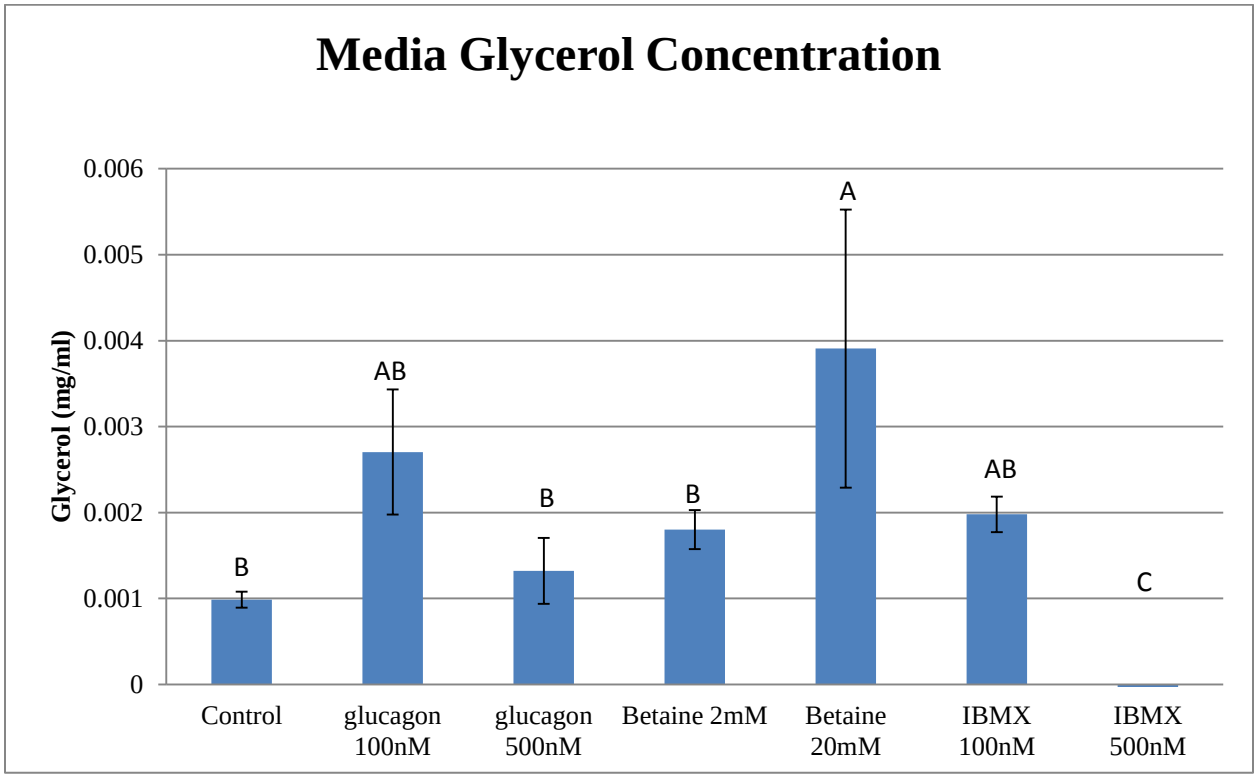

Figure 12. Glycerol concentration of explant media following one hour incubation
Effects of one hour treatment with lipolytic agents and betaine on media glycerol concentration (mg glycerol/ml media).Treatment details can be found in Table 1. Data are least square means and columns not sharing letters are different ($p<0.05$)

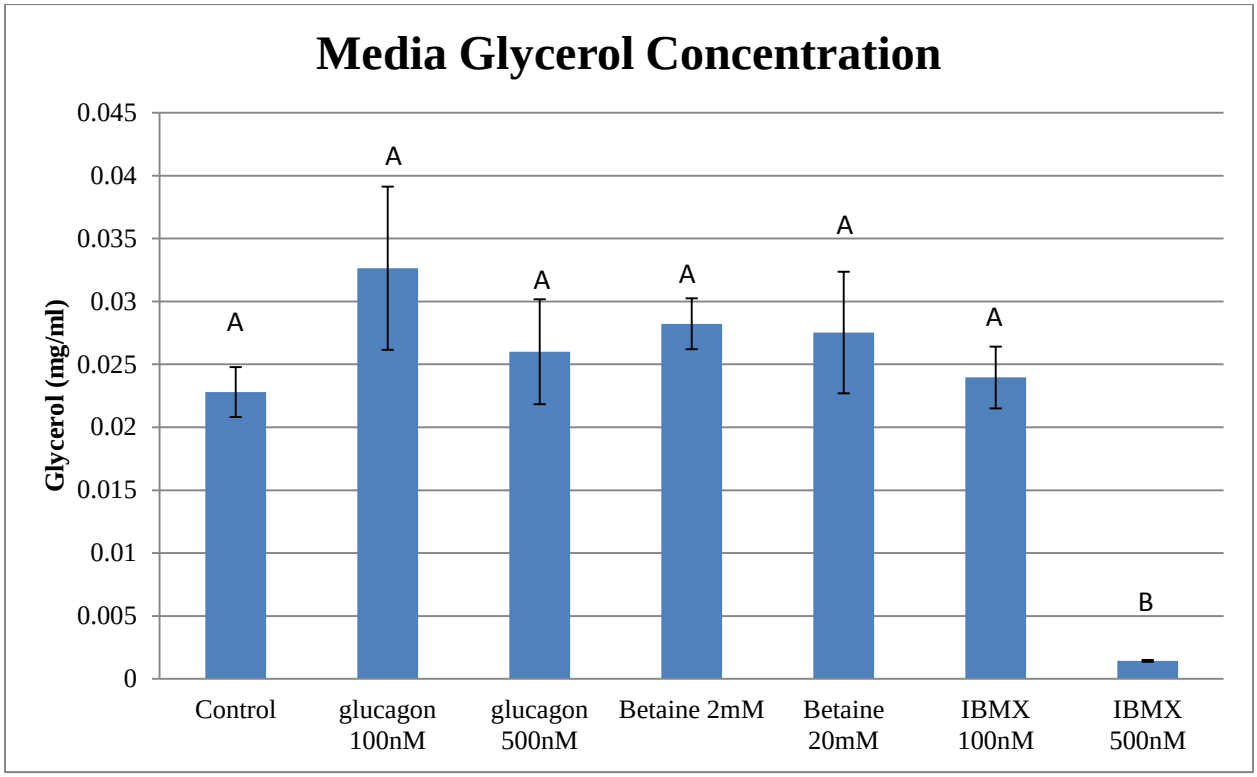

Figure 13. Glycerol concentration of explant media following twenty-four hour incubation Effects of twenty-four hour treatment with lipolytic agents and betaine on media glycerol concentration (mg glycerol /ml media). Treatment details can be found in Table 1. Data are least square means and columns not sharing letters are different ($p<0.05$)

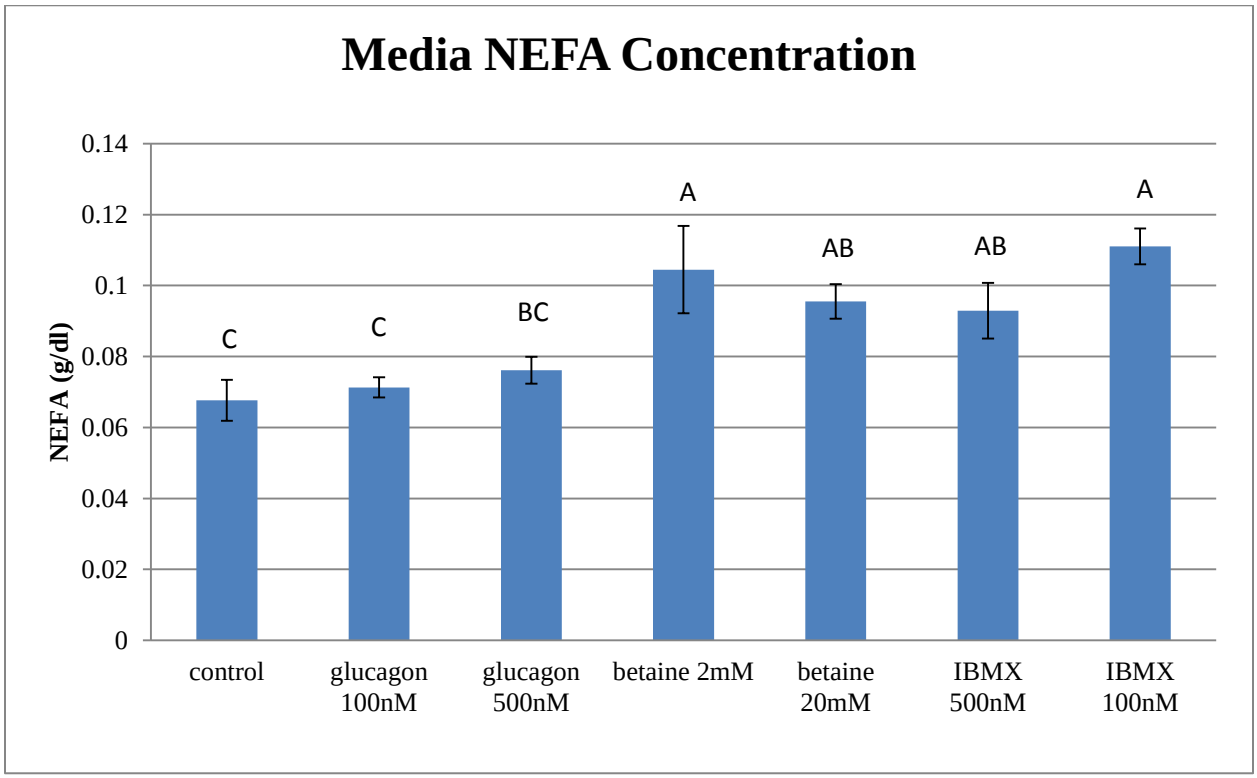

Figure 14. Effects of one hour treatment with lipolytic agents and betaine on media NEFA concentration. Treatment details can be found in Table 1. Data are least square means and columns not sharing letters are different ($p<0.05$)

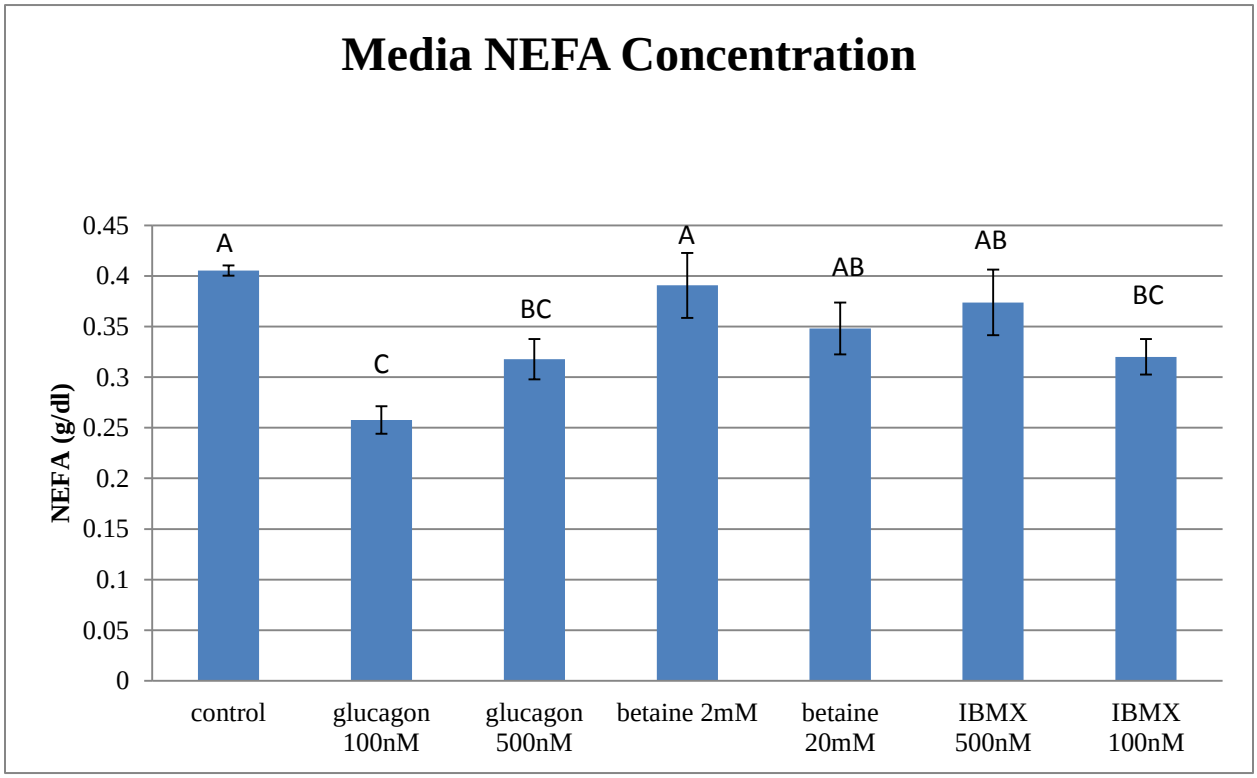

Figure 15. The effects of twenty four hour treatment of explants with lipolytic agents and betaine on media NEFA concentration. Treatment details can be found in Table 1. Data are least square means and columns not sharing letters are different ($p<0.05$)

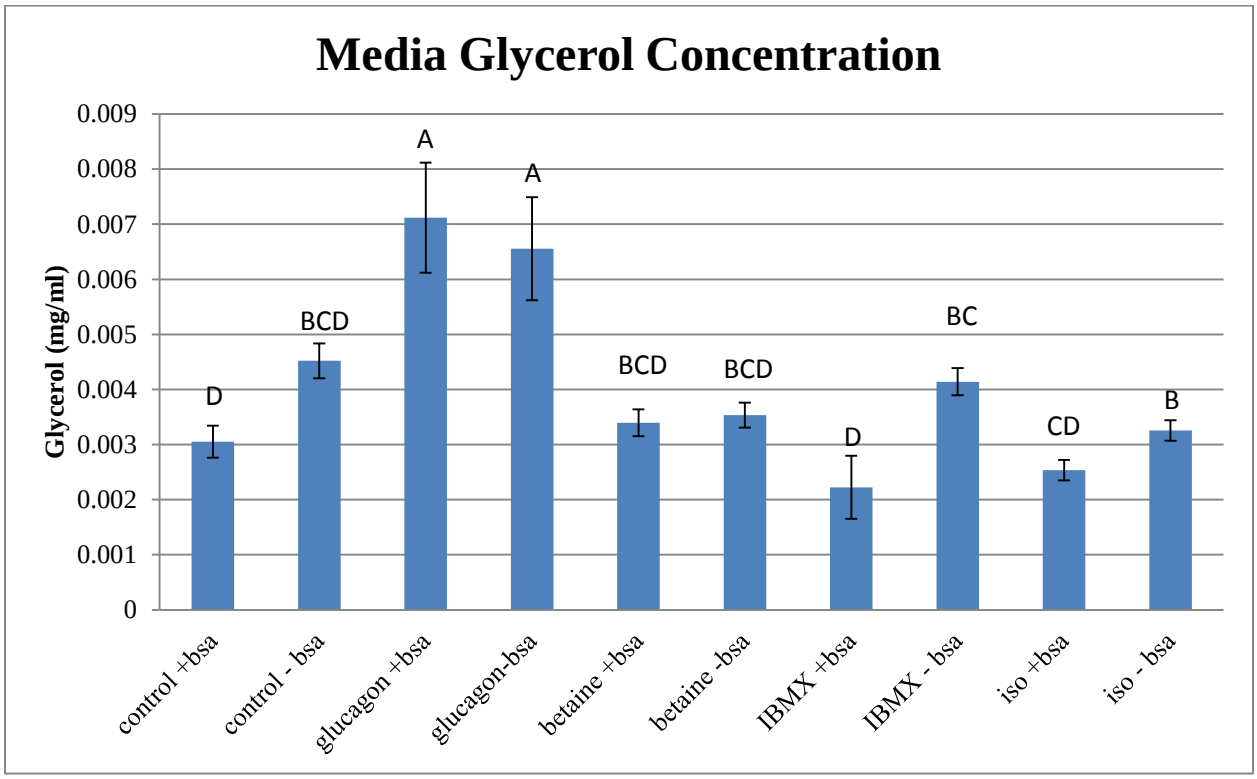

Figure 16. Effects of one hour treatment with lipolytic agents, with or without BSA (3%) and betaine on media glycerol concentration (mg glycerol/ml media). Treatment details can be found in Table 1. Data are least square means and columns not sharing letters are different ($p<0.05$)

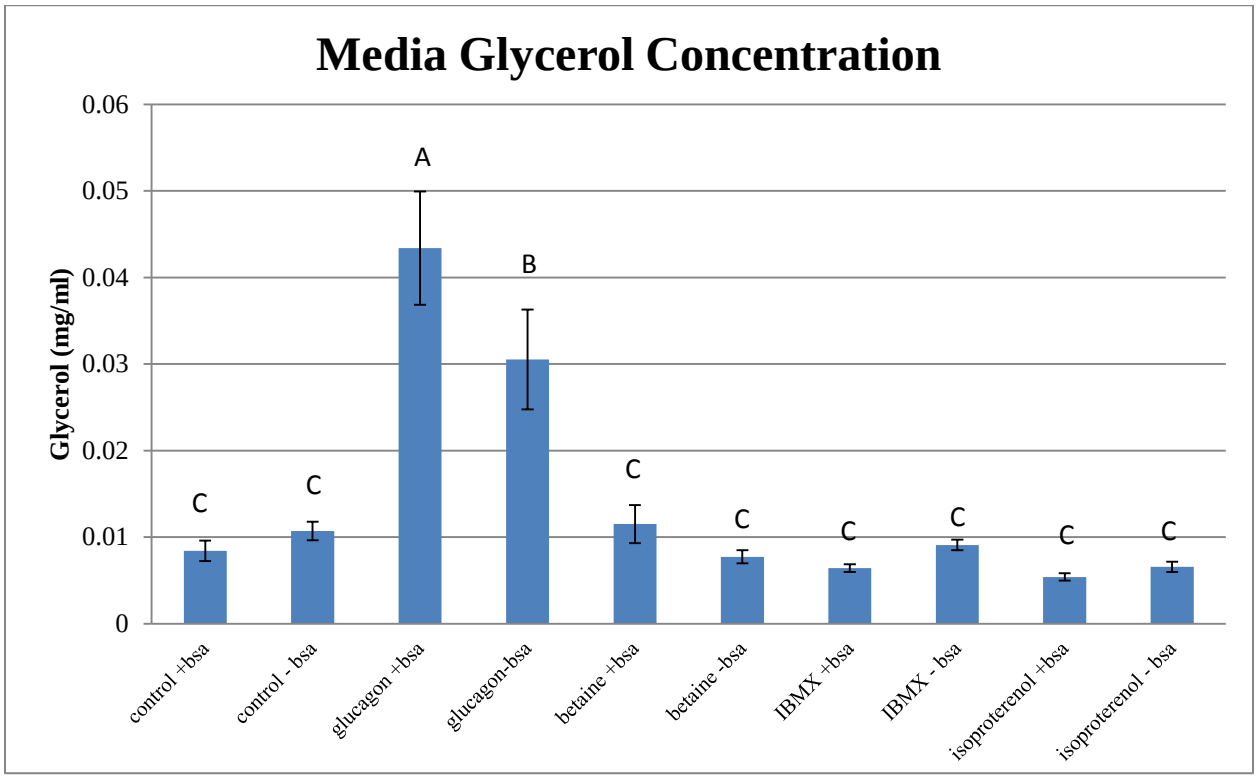

Figure 17. Effects of six hour treatment with lipolytic agents and betaine, with or without BSA (3%) on media glycerol concentration. Treatment details can be found in Table 1.IBMX; isobutyl-1-methylxanthine. Data are least square means and columns not sharing letters are different ($p<0.05$)

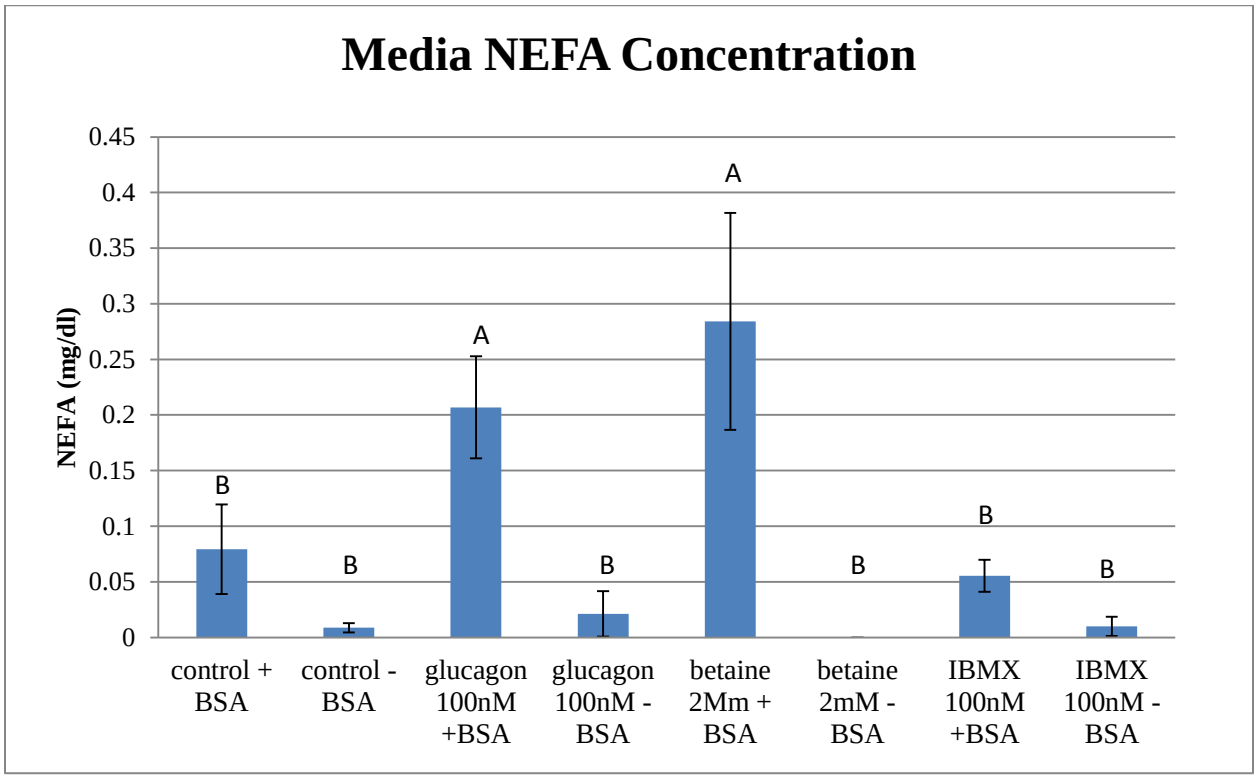

Figure 18. Effects of one hour treatment with lipolytic agents and betaine, with or without BSA (3%), on media NEFA (mg NEFA/ml media). Treatment details can be found in Table 1. Data are least square means and columns not sharing letters are different ($p<0.05$)

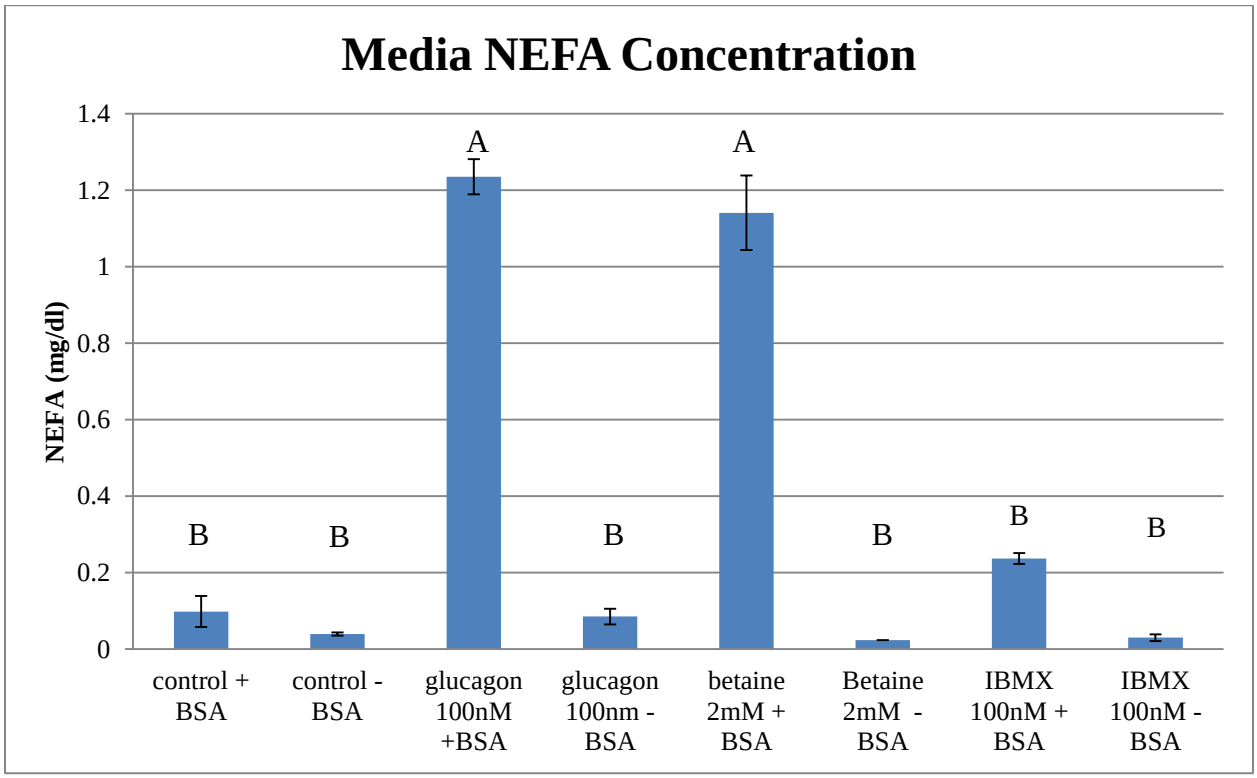

Figure 19. Effects of 6 hour treatment with lipolytic agents and betaine, with or without BSA (3%), on media NEFA concentration (mg NEFA/ml media). Treatment details can be found in Table 1. Data are least square means and columns not sharing letters are different ($p<0.05$

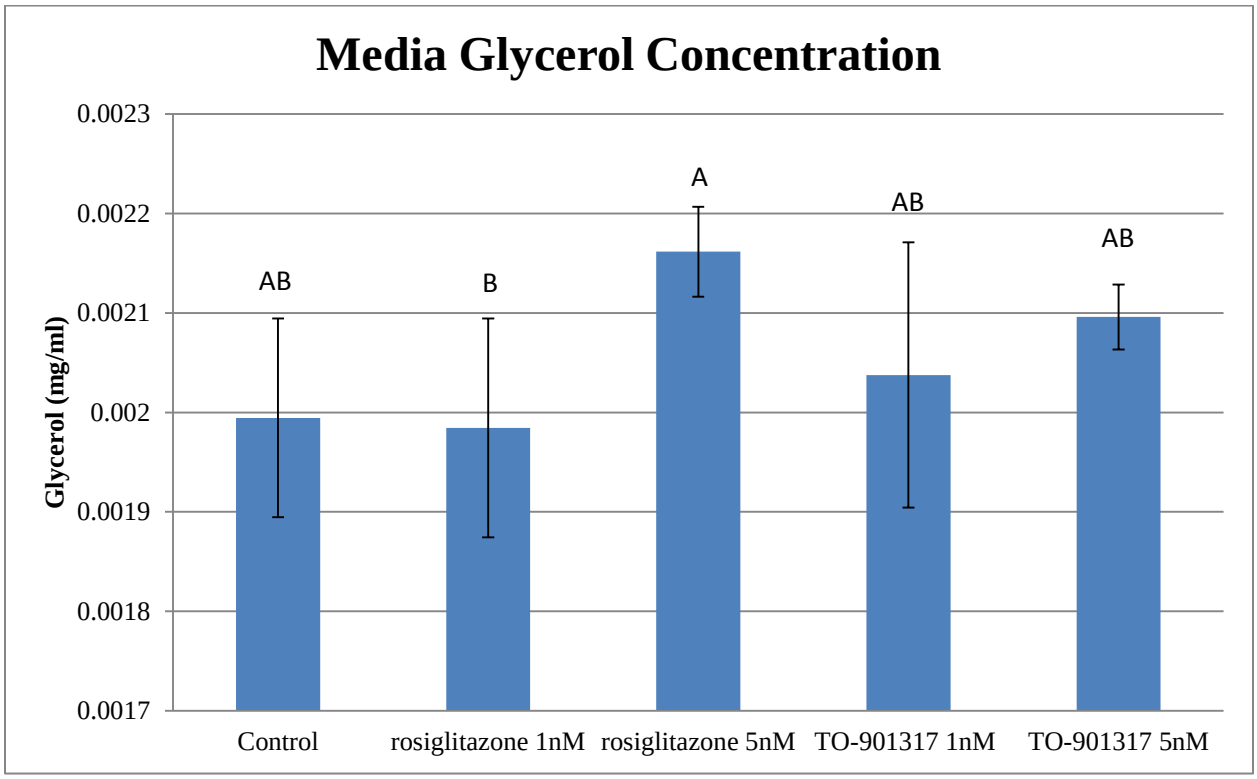

Figure 20.The effect of one hour treatment with nuclear receptors on media glycerol (mg glycerol/ ml media). Treatment details can be found in Table 1.Data are least square means and columns not sharing letters are different ($p<0.05$)

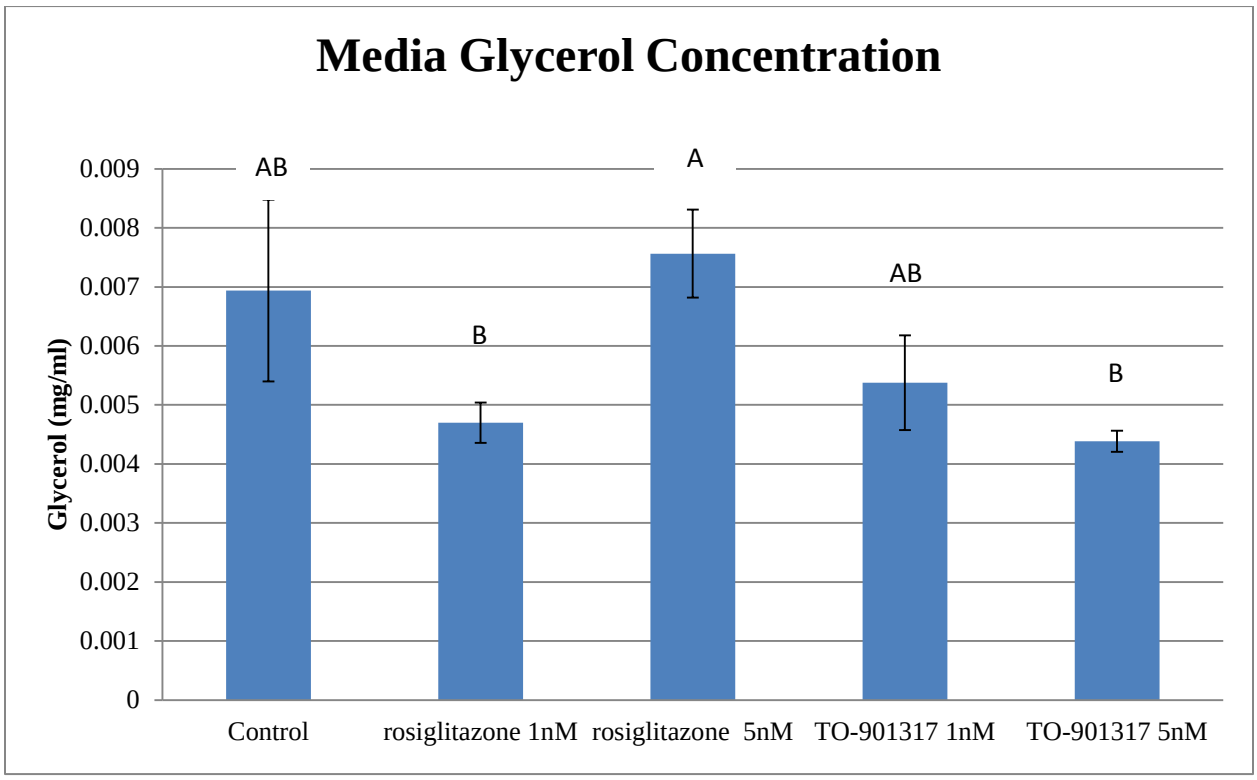

Figure 21.The effect of twenty- four hour treatment with nuclear receptor agonist on media glycerol (mg glycerol/ml media). Treatment details can be found in Table 1. Data are least square means and columns not sharing letters are different ($p<0.05$)

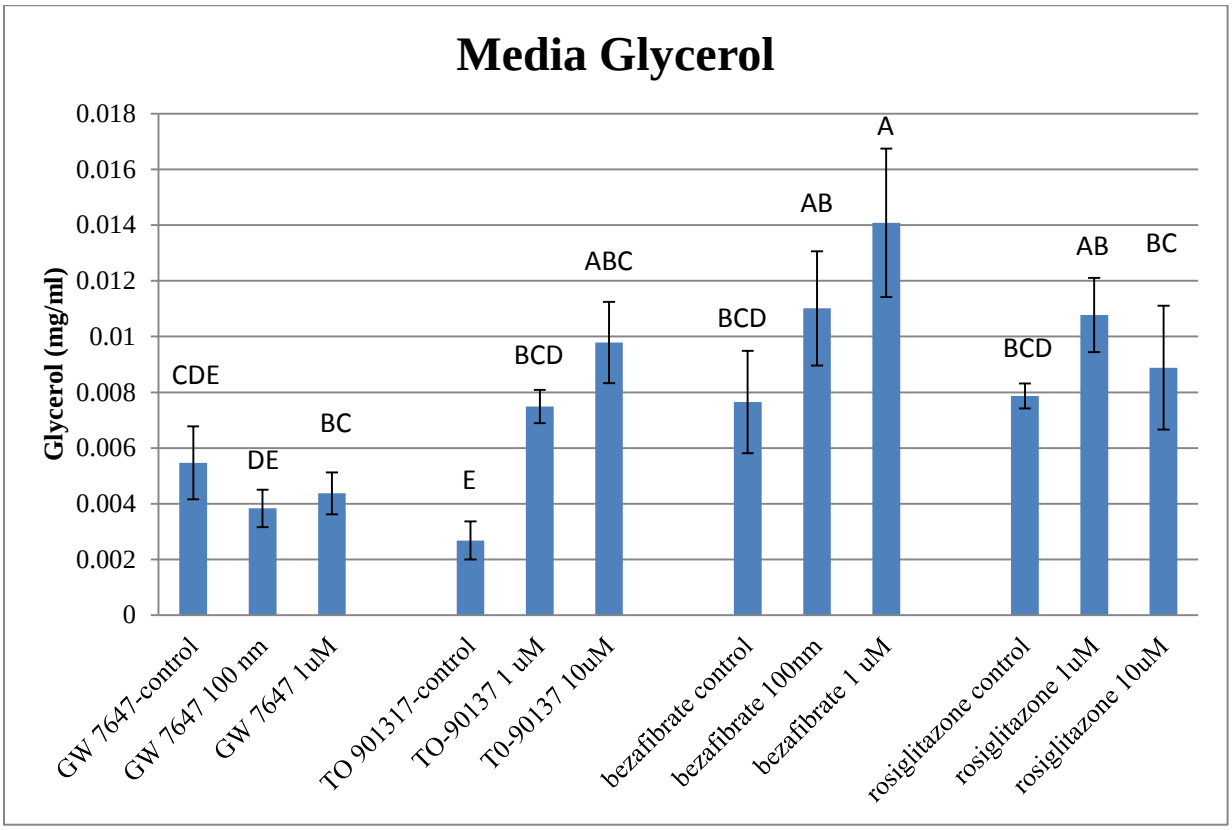

Figure 22. The effect of six hour treatment with nuclear receptor agonists on media glycerol (mg glycerol/ml media). Treatment details can be found in Table 1. Data are least square means and columns not sharing letters are different ($p<0.05$)

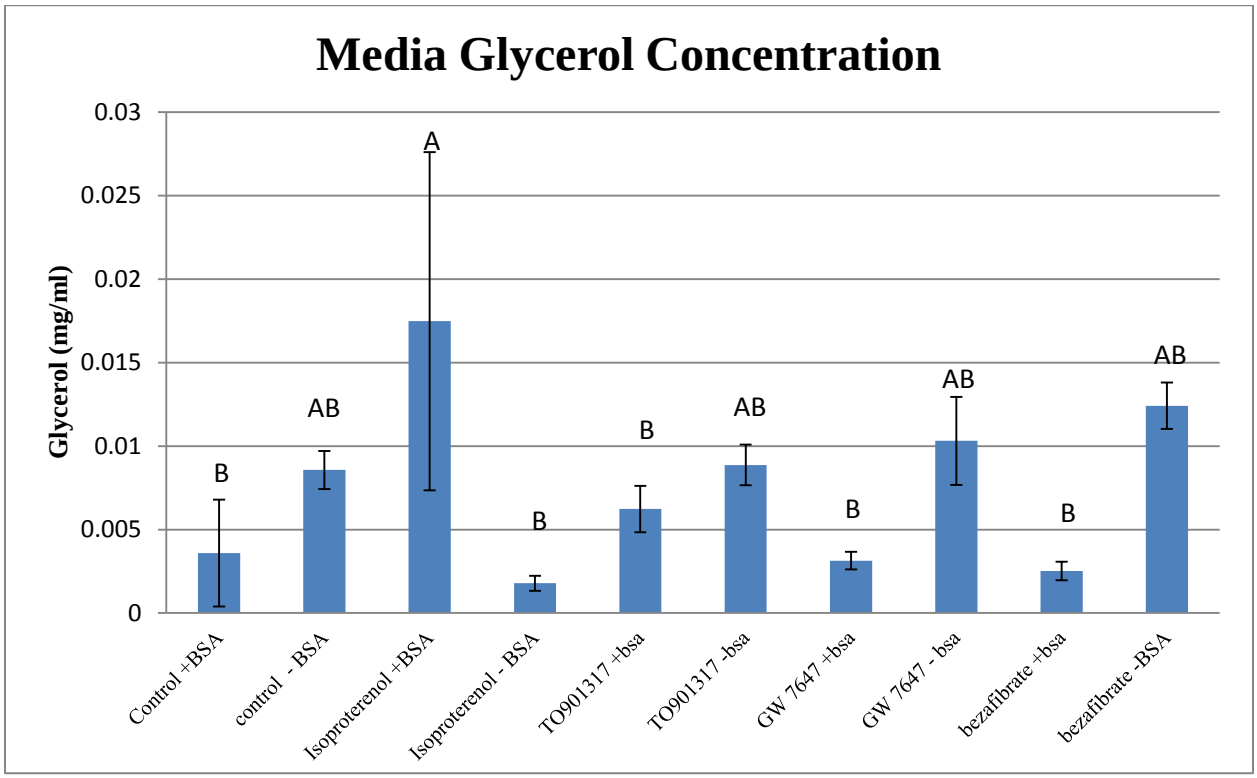

Figure 23. The effect of 24 hour treatment with nuclear receptor agonists on media glycerol (mg glycerol/ml media). Treatment details can be found in Table 1. Data are least square means and columns not sharing letters are different ($p<0.05$)

Chapter 5 Discussion

Betaine could prove to be an extremely useful tool in carcass modification as consumer wants and needs fluctuate. It appears as if manipulation of the cAMP dependent process of lipolysis is possible and relatively easy to accomplish. Diet is the most ideal method for initiation of lipolysis. With growing consumer consciousness associated with genetically modified organisms it is imperative that any act, intended to change the bodily composition of an agricultural animal, be received positively by the public. So long as wholesome foods and components are used any dietary treatment should fill this niche.

Betaine is attractive as a supplement because it occurs naturally in avian kidney cells as an osmoprotectant (Lien et al. 1993). Betaine can also spare if not replace methionine in the diet and has been shown to increase breast weight in broilers (Zhan et al. 2006). However, the mechanism by which betaine modifies the carcass is unknown. Betaine did not affect the carcass performance of broilers (Experiment 1) but there was an effect of betaine on circulating non-esterified fatty acids. The effect is unclear because the lower concentration of betaine caused a greater accumulation of NEFA than the higher betaine concentration (Figure 5). While betaine has been documented to reduce the adiposity of chickens (Zhan et al. 2006) itis unclear whether the fat was oxidized or deposited elsewhere. Itis possible that the increase in tissue weight is due to an increase in lipid content of those tissues. More probable is that the lipid is never transported from the liver. Therefore, it can be assumed that betaine's role in lipid metabolism exists in lipogenesis, transportation and storage rather than lipolysis and beta oxidation. If this is the case then betaine's use as a carcass modifier will depend on the fate of the animals. A deficiency in fatty acid transport could lead to a pathological increase in fat within the liver. In the case of

broilers, who have a relatively short life span, this would not be an issue, but an attempt to mimic BHMT deficiency in other animals that will live longer lives may be problematic.

3-Isobutyl-1-methylxanthine (IBMX) and betaine clearly have the greatest impact on lipid metabolism given the data presented. While IBMX is a noted phosphodiesterase inhibitor it is not yet known how betaine affects lipid metabolism. However, even though the mechanism is not understood it is clear that the end result, elevated NEFA concentration, is similar to glucagon. A mimicked response to a naturally occurring hormone is one step closer to a natural means of reducing adiposity. The nucleic receptors did not provide as drastic of a change in NEFA and glycerol concentrations observed with betaine and IBMX but, the results are still promising. TO-901317 and bezafibrate Table 1 exhibited the greatest effect on glycerol indicating an increase in lipolysis.

Lipolysis, the breakdown of triglycerides, results in the release of NEFA and a glycerol backbone. Elevated NEFA in circulation is attributed to leanness (Ji et al. 2012). In the studies described previously, certain treatments caused an increase in NEFA concentration. The drugs used as treatments are not ideal for achieving the goal of dietary manipulation using natural ingredients, but there are natural alternatives that can be used to obtain similar results. The success of IBMX as a stimulator of lipolysis indicates that phosphodiesterase inhibition maybe the lynchpin of lipolytic stimulation in the adipocyte. Caffeine, a natural phosphodiesterase inhibitor, is the first candidate that comes to mind for future studies. If added in the form of tea leaves or coffee, there exists the possibility for powerful natural stimulator of lipolysis.

It must also be considered that betaine may play a role in fatty acid synthesis. When insulin resistant HepG2 cells were treated with betaine at varying concentrations, AMPK phosphorylation was suppressed. This suggests that betaine might reduce hepatic fat synthesis (Kathirvel et al. 2010). Betaine hydroxy-methyl transferase deficiency leads to impaired triacylglycerol synthesis and enhanced glucose oxidation in isolated mature adipocytes, as well as, impaired triacylglycerol oxidation in liver homogenates (Teng et al. 2012).

In the case of TO-901317 and bezafibrate another ligand must be found for LXRα and PPARβ, respectively. The LXRα can be activated by oxysterols(oxidized cholesterol), and PPARβ can be activated via long chain fatty acids and eicosanoids. It can be assumed that supplementing the diet with either oxysterols, long chain fatty acids or eicosanoids will result in a similar response compared the supplementation of their pharmaceutical counterpart.

Lipotoxcity is the next obstacle to overcome if dietary carcass modification is to be achieved. In a normal fed animal, approximately 0.2% of endogenous fatty acids are oxidized, 50.1% are released, and 49.7% are re-esterified. Fasting doubles the partitioning of fatty acids toward oxidation in association with a 1.4-fold increase in lipolysis (Wang et al. 2003). The body is fine tuned to these specifications and an increase in lipid within tissue can lead to cellular dysfunction and apoptosis (Schaffer 2003). Therefore, the fate of free fatty acids following induced lipolysis is of concern. Overloading of the oxidative machinery could result in increased re-esterification which is counterproductive if the end goal is reduction of whole body lipid composition.

The real world possibility of using lipolysis as a tool to reduce adiposity in broilers is high. It is clear from the data that manipulation of the cAMP cascade can induce a lipolytic

state, and it is also clear that LXRα and PPARβ also play a role in the regulation of lipolysis. While these statements have been validated in vitro it is still unclear what happens in the whole bird. To test this, an ambitious, multifaceted live bird trial would be ideal. Such an experiment would be performed, as follows:

First, a small pilot study using a small number of chicks will be performed. The chicks would be slaughtered and their fat will be harvested and cultured using the protocol proposed previously. The fat will be treated using IBMX, TO-901317, and bezafibrate, just as before. However, caffeine, and a cocktail of long-chain polyunsaturated fatty acids would also be used as treatments to ascertain their efficacy in vitro. Second, assuming that caffeine and the long chain polyunsaturated fatty acids are effective substitutes for IBMX and the nuclear receptor agonists. A large scale broiler study using one-thousand birds per treatment will be implemented. Proposed treatments are as follows:

1. Control - basal diet with choline level formulated to NRC level
2. Betaine - basal diet supplemented to provide 0.6g/kg of betaine. This diet will assess the ability of betaine to serve as a method to augment lipogenesis and transport
3. PUFA- basal diet supplemented with a source of long chain poly unsaturated fatty acids. Examples of sources include: flax seed, safflower oil and fish oil. This diet will used as an activator for Liver X Receptor
4. PUFA + Betaine- basal diet supplemented with both betaine and a PUFA source. This diet will be used to test if there is a compounded effect of feeding a PUFA source and betaine.

Phase feeding will be utilized and samples will be taken at each phase. Blood will be used to measure NEFA, glycerol, glucose and triglycerides. Liver biopsies will be taken to measure fatty acid content of the liver this will serve as an indication of fatty acid delivery. Fat

biopsies will be used histologically to measure adipocyte size to approximate the amount of lipid being stored by the birds. Fat and liver will be snap frozen for future analysis of gene expression primarily those associated with the Liver X receptor. Performing these tests at the changing of the dietary phases will create a lipolytic time line for the lifetime of a broiler allowing producers to pinpoint the ideal time for supplementation.

At the finisher phase a caffeine source will be added to test the efficacy of caffeine as a lipolytic stimulus. The reason for adding caffeine at the end is because it is assumed that caffeine will be the most powerful "fat burner". The finishing phase is when the birds are prepared for slaughter and if the birds are too lean during other phases, growth and flock health could be compromised.

Slaughter provides the opportunity to study the effects of these diets on a fasted bird as industry recommends a twenty four hour fast before slaughter. At slaughter a live weight will be taken prior to kill. This allows for dressing percentage to be analyzed. Additional carcass measurements will include: wing weight, breast percentage and leg quarter percentage. Muscle will also be tested for enrichment of polyunsaturated fatty acids. Blood will be taken again for post mortem analysis NEFA, glycerol, glucose and triglycerides. Fat and liver will be harvested weighed and snap frozen for analysis of gene expression.

The future for the use of lipolysis as a tool for carcass modification is bright and success of such a large scale experiment would cement the use of lipolysis as a means to improve both profitability and marketability of the chicken. Proving the efficacy of caffeine and long chain PUFAs will not only reduce the adiposity of the chicken, but if the meat is enriched with long chain PUFAs chicken meat can be marketed as a healthy option much like eggs or milk enriched

with omega-3 fatty acids. There would also be an increased demand in caffeine(tea or coffee), flax, or safflower seeds adding value to these commodities as well.

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Vita

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