Manipulating Adipose Tissue Fatty Acid Oxidation to Reduce Fatness in Broiler Chickens

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ABSTRACT

Compared to rodents, broiler chickens, those reared for meat, are an attractive model for studies of adipose biology, and obesity development in children. The broiler chicken lacks the gene for uncoupling protein 1, the hallmark for brown adipose tissue making them a useful model to study lipid metabolism in white adipocytes. Two studies were performed to investigate if white adipose tissue had the metabolic ability for fatty acid oxidation (FAO), and to investigate the effects of dietary fatty acids on abdominal fat development of young broiler chickens as a model for childhood obesity. In study one, chickens were fasted for three, five, and seven hours. Afterwards, the oxidative flux from the citric acid cycle, and the citrate synthase enzyme activity were measured in white adipose tissue. In study two, young Cobb500 broilers, from age seven to 21 days, were fed isocaloric diets prepared using lard (primarily saturated), corn oil (primarily monounsaturated), flaxseed oil (enriched in alpha linolenic acid (ALA, 18:3, n-3)), or fish oil (enriched in eicosapentaenoic acid (EPA, 20:5, n-3) and docosahexaenoic acid (DHA, 22:6, n-3)), at 8% fat by weight. Physical characteristics, abdominal adipocyte histology, and abdominal adipose tissue gene expression profiles were altered due to dietary fatty acids. Collectively our studies confirm that white adipose tissue has the capacity to increase local FAO by increasing expression of key regulatory enzymes and proteins. Further, by altering the type of fatty acids consumed during childhood, adipose deposition

and adipocyte size can be attenuated. These data confirm that FAO can be induced locally in white adipose tissue, dietary long chain n-3 polyunsaturated fatty acids promote reduced adipocyte size, and finally that these data could offer new therapeutic targets to reduce fatness in chickens and children.

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CHAPTER I
LITERATURE REVIEW

1.1 Obesity

Adipose tissue is specialized tissue designed to store excess energy in the body. When energy intake and expenditure are in balance, adipose tissue mass is maintained at a steady level. Once energy is in excess, due to either increased intake or reduced expenditure, it is stored in adipose tissue in the form of triacylglycerol (TG) molecules. These molecules are made of three fatty acids esterified to a glycerol backbone. Adipose mass expands as TG storage increases, once energy intake consistently exceeds expenditure. However, when demand for energy increases or energy intake is low, fatty acids are hydrolyzed from the glycerol backbone and released into circulation for use by other tissues.

Obesity is a condition of excess adipose tissue. Clinically, obesity is diagnosed by defining body mass index (BMI) (kilogram body weight/squared height in meters), and individuals having a BMI > 30 kg/m^2 are considered to be obese. Obesity is considered to be an epidemic, and is prevalent in the United States and abroad. Presently, the United States Centers for Disease Control and Prevention (CDC) reports that 34.9% of the adult population and 16.9% of children and adolescence in the United States are obese (Ogden *et al.*, 2014). Obesity manifests as excessive weight gain and contributes to a number of

serious medical conditions such as sleep apnea, asthma, cancer, cardiovascular disease, type 2 diabetes, nonalcoholic fatty liver disease, infertility, hypertension, and orthopedic complications (Daniels *et al.*, 2005).

Excess lipids in circulation, also known as hyperlipidemia, changes the blood pH, impairs cardiac efficiency, promotes inflammation, and reduces insulin sensitivity. All of which contribute, at varying levels, to obesity associated comorbidities because an overabundance of fat, as is present in obese individuals, renders adipose tissue dysfunctional by weakening its ability to properly control the balance of fats in circulation and storage. The incidence of obesity is predicted to continue a rising trend, making health costs associated with its treatment an economic burden (Nielsen *et al.*, 2014).

Factors that contribute to obesity are genetics, diet, and lifestyle. To assess the genetic influence on increased adipose mass in developing children, one study identified influential single nucleotide polymorphisms (SNPs) among twins (Llewellyn *et al.*, 2013;2014). Of mothers who were obese pre-pregnancy, Rooney *et al.* (2011) found that more than half of their offspring developed obesity in childhood, adolescence, and 44% in early adulthood when comparing gestational weight gain, maternal obesity, weight gain in infancy, and weight gain in early adulthood. Investigating the influence of lifestyle, another study reported 64.6% of the subjects who were obese or overweight in childhood became obese adults; surmising that obesity in adulthood is frequently preceded by obesity during childhood (Juonala *et al.*, 2011).

1.2 Adipose tissue biology

Adipose tissue is comprised of adipocytes and vascular stroma cells. Adipocytes are specialized energy storage cells that help maintain whole body energy homeostasis through the storing and release of lipids from lipid droplets. Vascular stroma cells in adipose tissue are pre-adipocytes, fibroblasts, and varying immune cells. Adipocytes can be found throughout the body, including in muscle and breast tissue, but most readily found in adipose tissue depots: subcutaneous depot, and visceral depot. Histologically, adipocytes occupy virtually all of adipose tissue, however Tchoukalova *et al.* (2004) reported the proportion of adipocytes versus vascular stroma cells in adipose tissue of obese patients to be approximately 20% to 40% depending on the depot, and 35% to 45% of the vascular stroma cells were pre-adipocytes.

The capacity of adipocytes to oxidize fat is associated with the amount of mitochondria within adipocytes, and serves to distinguish name: white, beige, and brown. White and beige adipocytes store lipids in one large lipid droplet, however beige adipocytes contain more mitochondria than white adipocytes. Contrasting characteristics of brown adipocytes versus white and beige are: many smaller lipid droplets, an abundance of mitochondria, require more oxygen, and have the highest capacity for fatty acid oxidation (Harms *et al.*, 2013; Rosen *et al.*, 2014).

The lipid droplet is a specialized organelle with a phospholipid membrane encasing the stored sterol esters and TG within adipocytes. Adipocyte size is determined

by the lipid droplet size, which effects adipocyte expandability and functionality. Through signaling cascades that support energy demand, lipid droplets release TG molecules, which causes an associated reduction in adipocyte size. In response to excess energy, lipid droplets accommodate TG storage, increasing lipid droplet and adipocyte sizes (Fruhbeck *et al.*, 2014). Obesity causes lipid droplets to over expand to accommodate the excess TG that require storage, however large adipocytes are more resistant to cellular signaling which negatively impacts the balance energy (Lofgren *et al.*, 2005).

Adipocytes are derived from mesenchymal stem cells (MSCs) that can develop into chondrocytes, osteoblasts, myocytes, or adipocytes. To become mature adipocytes, MSCs must first commit into pre-adipocytes. Pre-adipocytes cannot be reverted, and regulation of adipocyte differentiation is through insulin, glucocorticoids, cyclic AMPs (cAMP) stimulations, and peroxisome proliferator activated receptor gamma (PPARγ) (Henry *et al.*, 2012). Pre-adipocyte maturation cascades through the transcription factor signaling of members of the AP-1, Kruppel-like factors (KLF), CCAAT-enhancer-binding proteins (C/EBPs), zinc finger protein (ZFP), and sterol regulatory element-binding proteins (SREBP), and then by activating PPARγ (Rosen *et al.*, 2006; Stephens, 2012). The most important regulator of adipogenesis is PPARG, as it is essential for promoting and maintaining the differentiated state, and can drive non-adipogenic cells, such as fibroblasts and myoblasts, into becoming adipocytes (Rosen *et al.*, 2014).

Adipocyte TG level is regulated by the balance between anabolic hormones that promote energy storage, and catabolic hormones that promote energy utilization. Insulin is the primary anabolic hormone, and glucagon is the primary catabolic hormone. Insulin promotes lipid storage by stimulating adipocytes to uptake glucose for lipogenesis, de novo fatty acid and TG synthesis, which can also take place in the liver. Insulin inhibits lipolysis, the mobilization and release of fatty acids into circulation. Glucagon is catabolic because it stimulates lipolysis (Nuttall *et al.*, 2015; Olefsky, 1976; Randle *et al.*, 1963).

In times of energy surplus insulin stimulates properly functioning adipocytes to store lipids as tri-, di-, and mono- glyceride molecules, which are fatty acids esterified to glycerol, which are products of adipocyte glucose metabolism. In times of energy demand, e.g. during fasting, glucagon and other catecholamine molecules stimulate adipocytes to free fatty acids into circulation. The lipolysis cascades with lipase enzymes that hydrolyze tri-, di-, and mono- glyceride molecules into non-esterified fatty acids (NEFA) and a glycerol backbone. Lipid storage, lipogenesis, and lipolysis are stimulated in adipose tissue to maintain the balance between lipids and carbohydrates. This important and highly regulated homeostasis functions to combat against high blood glucose and hyperlipidemia (Lass *et al.*, 2011).

In addition to its role in energy storage, adipose tissue also acts as an endocrine tissue by synthesizing and releasing proteins and peptides, referred to as “adipokines.” The term adipokines is derived from “adipose cytokine” because many adipokines are

classical pro-inflammatory cytokines such as tumor necrosis factor alpha (TNFα). Adipokines are essential in the regulation of metabolism. Adiponectin, an adipokine with endocrine function, improves energy metabolism in tissues by increasing insulin sensitivity, which additionally inhibits lipolysis and lipogenesis. Both, insulin and adiponectin work in tandem to help adipose tissue regulate energy homeostasis (Henry *et al.*, 2012). Obesity negatively impacts adipose tissue adipokine production and function, because lipid droplet size is inversely correlated with proper adipocyte function. A superfluity of TG impedes the production of adiponectin, leading to insulin insensitivity and homeostatic imbalance between lipolysis and lipid storage (Henry *et al.*, 2012; Qiao *et al.*, 2011).

Further, adipose tissue helps regulate energy intake by secreting leptin, a signaling molecule produced by adipocytes that suppresses appetite (Harris, 2014). A study investigating if leptin production became normal post obesity reported a relative deficiency of leptin in the adipose tissue of post obese women. The low basal leptin production was attributed to the positive relationship between adipocyte volumes and leptin secretions (Lofgren *et al.*, 2005). This is consistent with an in vitro study using mice that showed mRNA levels of leptin were positively correlated with both leptin secretion and adipocyte volumes in inguinal, epididymal, and retroperitoneal adipose depots (Zhang *et al.*, 2002). Although there was no change reported in the mass of adipose tissue

of post obese women, hypercellularity was reported (Lofgren *et al.*, 2005), which suggests that the observed weight loss may have affected fat cell size rather than fat cell number.

The number of adipocytes is also intimately linked with energy balance. A recent investigation reported that obese adults had a greater number of new cells and a greater number of adipocytes than lean adults, although both had an approximate 10% adipocyte turnover every year. The weight loss in obese adults was reflected in the fat cell volume while the fat cell number remained invariable in post obese adults. Overall, this study confirmed that energy balance is also associated with the number of adipocytes. Further, they determined that adipocyte number is strictly regulated and static in adulthood, and this number is potentially fixed during childhood and adolescence (Spalding *et al.*, 2008).

Previous studies have reported that obese children are much more likely to become obese adults, adipose tissue hypercellularity impedes appetite suppression, and adipocyte number is static in adulthood, and potentially predetermined during childhood and adolescence. Together these studies reveal the need for childhood obesity research to provide interventions for adult obesity preventions, and bares factors that contribute to the difficulties obese adults face in maintaining weight loss (Juonala *et al.*, 2011; Lofgren *et al.*, 2005; Spalding *et al.*, 2008). Overall, understanding the underlying mechanisms with which adipose tissue functions to regulate energy metabolism will provide valuable insight to lighten the associated health and economic burdens caused by obesity.

1.3 Fatty acid metabolism

Fatty acids are composed of an aliphatic acyl chain tail and a carboxylic acid head. They are classified by length of the fatty acyl tail, presence or absence of double bonds, and the location of the first double bond. The length of fatty acids are termed short, medium, long, and very long, having ≤ 6, 8 to 14, 16 to 22, or ≥ 22 carbons respectively. The levels of unsaturation for fatty acids are saturated (SFA) and possess no double bonds, mono-unsaturated (MUFA) and hold only one double bond, or poly-unsaturated (PUFA) and possess multiple double bonds. The occurrence of the first double bond from the methyl end of the aliphatic tail establishes the omega number as omega 3 (n-3), omega 6 (n-6), omega 9 (n-9), etc. which occur on the third, sixth, or ninth carbons respectively.

In some organisms, the levels of essential fatty acids, which are n-6 and n-3 long chain poly-unsaturated fatty acids, are controlled by the diet due to impaired synthesis of the production of fatty acids longer than 16 carbons. Notable long chain n-3 poly-unsaturated fatty acids (n-3 PUFA) are: alpha-linolenic acid (ALA) (18:3 n-3), eicosapentaenoic acid (EPA) (20:5 n-3), docosahexaenoic (DHA) (22:6 n-3), having 18, 20, and 22 carbons respectively with 3, 5, and 6 double bonds respectively. Long chain n-6 poly-unsaturated fatty acids (n-6 PUFA) that are noteworthy are: linoleic acid (LA) (18:2 n-6), gamma linoleic acid (GLA) (18:3 n-6), and arachidonic acid (AA) (20:4 n-6), having 18, 18, and 20 carbons respectively with 2, 3, and 4 double bonds respectively.

Fatty acids are the most energy dense substrates by providing nine calories per gram, which is more than any other energy molecule, i.e. proteins, carbohydrates, and alcohols. Fatty acids are required by nearly all tissues and cell types to use as metabolic fuels, signaling molecules, and cellular membrane components, and long chain PUFAs function in a number of mechanisms including adipose tissue metabolism. Both n-3 PUFA and n-6 PUFA are precursors for eicosanoid molecules, which are the primary drivers of the body's inflammatory responses, but n-6 PUFA have contrasting physiological functions to n-3 PUFA. Anti-inflammatory eicosanoids are derivatives of the n-3 PUFA EPA, while pro-inflammatory eicosanoids are derivatives of the n-6 PUFA AA. These fatty acids are found abundantly among phospholipids of cell membranes, and compete for positions on phospholipids. When cleaved from phospholipids, EPA or AA are released. Notably, AA is a precursor of pro-inflammatory prostaglandins, and EPA is a precursor for anti-inflammatory prostaglandins (Ferrero-Miliani *et al.*, 2007).

Saturated and n-6 PUFA are commonly found in animal fats and vegetable oils, and n-3 PUFA are prevalent in flaxseeds, walnuts, or fresh water fishes (Ailhaud, 2005; Sears *et al.*, 2011). Lard and cheap vegetable oils are often used in the production of the most prevalent and readily available processed foods. This helps to clarify the prevalence of SFA and n-6 PUFA rather than n-3 PUFA in the average US consumer diet (Sears *et al.*, 2011). Therefore, it is no wonder that overweight, obesity, and comorbid conditions are popular in the United States (Kopecky *et al.*, 2009).

Dietary PUFAs may play a role in metabolism by stimulating lipid consumption. Despite double bonds being stronger than single bonds, double bonds are less stable and more reactive, rendering unsaturated fatty acids more vulnerable to oxidation compared to saturated fatty acids. For example, a reduction in fatness and improved glucose metabolism was observed in humans and other mammals fed diets enriched with n-3 PUFA versus n-6 PUFA as dietary supplements. This gives rise to a potential nutritional application that could reduce the incidence of obesity (Kopecky *et al.*, 2009).

Fatty acid oxidation occurs in mitochondria and peroxisomes to supply cells with energy in the absence of glucose. Furthermore, fatty acid oxidation is regulated by glucagon and insulin, which are controlled by nutritional intake, in properly functioning organisms. Mitochondria can oxidize short, medium, and long chain fatty acids, and peroxisomes can oxidize very long chain fatty acids that are then supplied to mitochondria. Carnitine-palmitoyl transferase 1a (CPT-1a), the rate limiting enzyme in mitochondrial fatty acid oxidation, transports fatty acids that have been attached to coenzyme A (CoA), into the mitochondria. Once inside the mitochondrial matrix, the fatty acyl-carnitine (such as palmitoylcarnitine) reacts with coenzyme A to release the fatty acid for breakdown into acetyl-CoA. The rate limiting enzyme in peroxisomal fatty acid oxidation is acyl-CoA oxidase 1 (ACOX1), which is the first enzyme of the fatty acid oxidation pathway in peroxisomes. In peroxisomes, very long chain fatty acids are broken down into shorter chained fatty acids, which can be oxidized by the mitochondria.

Having a capacity for fatty acid oxidation is not a characteristic commonly attributed to white adipocytes. However, recent studies have verified otherwise, highlighting a potential pathway in white adipocytes to oxidize fatty acids locally (Ji *et al.*, 2012), which may suggest that accumulating fat excessively is impeded in the presence of PUFA because dietary PUFAs may reduce fatness by stimulating lipid clearance and fatty acid oxidation. Mechanistically, dietary n-3 PUFAs may enter cells of the liver and skeletal muscle, and act as ligands for nuclear receptors that go on to increase transcription for fatty acid oxidation, and glycogenesis. This increase in lipid oxidation potentially leads to a reduced amount of fatty acids in circulation destined for storage in adipose tissue, which then reduces adipocyte volume. By identifying and characterizing the regulatory pathways for lipid metabolism in visceral white adipose tissue, and determining the effects of nutritional approaches through diet supplementation with n-3 PUFAs, the present study will be useful in making an impact in obesity research.

Peroxisome proliferator–activated receptors (PPARs) are nuclear receptors that regulate the expression of respective genes, which control lipid and glucose homeostasis. Three distinct PPARs have been discovered: PPARα, PPARβ/δ, and PPARγ. The alpha type, β/δ, γ2, and γ3 are expressed in adipose tissue where their primary functions have been characterized as regulators of both glucose and lipid homeostasis (Xu *et al.*, 1999). Peroxisome proliferator–activated receptors gamma is pivotal in adipogenesis, as it is required for the transcriptional events through which pre-adipocytes (a fibroblast-like

cell) differentiate into mature adipocytes. The gamma receptor is also responsible for keeping adipocytes in the differentiated state (Rosen *et al.*, 2006). In times of low energy, PPARα, predominantly found in the liver, helps to regulate lipid homeostasis by stimulating fatty acid uptake (Forman *et al.*, 1997; Lee *et al.*, 2003). The activation of PPARβ/δ, found ubiquitously, may function to regulate circulating cholesterol levels and insulin sensitivity (Lee *et al.*, 2006; Mottillo *et al.*, 2012)

Research has suggested that sensing and regulating lipid levels is a major function of PPAR family nuclear receptors (Royan *et al.*, 2011). Lipid metabolism in adipose tissue is effected by long chain polyunsaturated fatty acids. Xu *et al.* (1999) reported that EPA, a long chain n-3 PUFA, acted as the primary ligand to activate PPARβ/δ. Then, using labelled ligands in competitive binding assays, Wahle *et al.* (2003) confirmed that PUFA can function as activating ligands for PPARs. Overall, n-3 PUFA can act as ligands for nuclear receptors, promote anti-inflammatory cytokines, and, due to the reactive double bonds, encourage lipid oxidation, all of which discourages fat deposition.

1.4 Broiler Chickens

The National Chicken Council (2014) reports that poultry is the number one protein source consumed in the United States. The commercial poultry industry raises chickens for two major purposes, which are layers or broilers. Layers are reared to reach

sexual maturation to produce eggs, and broilers are raised for their body meat. In 2014, 84.7 pounds per capita of chicken was consumed in the US alone, which is more consumed chicken than any other country in the world, and according to the National Chicken Council (2014), the increasing consumer demand for chicken is present in both the US and internationally. This is perhaps due, in great part, to increasing population, cost comparison between chicken and other meats, life expectancy, as well as the increasingly health conscious consumers choosing leaner white meat, over red meats.

Through phenotypic selection for rapid growth, due to rising consumer demand, broiler chickens have become fatty (Collins *et al.*, 2014). As with many species, humans included, excessive fat can cause a great deal of complications in organisms. This fatness effects the poultry industry by rendering fatty broilers less marketable as consumers view fatness as unattractive, and by negatively effecting fertility in layers. Fatness can manifest in layers by causing decreased egg production with two or more ova on the same day. This then leads to a high proportion of unmarketable eggs with defective shells (Griffin *et al.*, 1994).

Feeding chickens accounts for 60 to 75% of the total cost of poultry production (Chiba, 2014). A common approach to the issue of fatness in chickens has been to reduce the caloric intake, which is similar to the obesity approach in humans. However, reducing caloric intake to decrease fatness in chickens has undesirable effects on the energy and nutrient requirements necessary for sustaining growth. For example, a study using older

broiler chickens aged 18 to 53 days reported the greatest difference in abdominal fat pad weights with an associated decreased body weight gain and decreased feed conversion ratio when the daily metabolizable energy was reduced by 310 kilocalories/kilogram (kcal/kg), while supplementing L-carnitine in the diets (Rabie *et al.*, 1998). A reduced caloric intake decreases meat yield, which has a negative impact on the poultry industry (Chiba, 2014). Therefore, producing applicable approaches to reduce fatness, and siphon fat deposition in broilers has economic value for the poultry industry.

Nutritional factors, such as dietary PUFA, have been shown to reduce fatness in adult humans (Harden *et al.*, 2014; Munro *et al.*, 2013). Due to its importance for maintaining growth, some studies have emphasized diet formulation as the prime alternative to combat fatness in chickens. There have been few studies examining the effects of dietary n-3 PUFAs on broiler chickens. One study reported lowered total body fat and a higher rate of fat oxidation in broiler chickens consuming dietary fat as sunflower oil versus those supplemented with saturated animal fats (Sanz *et al.*, 2000). In a study where chicken diets were supplemented with fish oil, sunflower oil, or tallow, there was a reported significant reduction in abdominal fat percentage in those that consumed PUFAs vs. saturated fats (Newman *et al.*, 2002). All in all suggesting that PUFAs may act to induce lipid metabolism in white adipose tissue of both humans and broilers.

The chicken is an underutilized model for human obesity research. In chickens, lipogenesis primarily occurs in their liver, they are naturally hyperglycemic, insulin

resistant, and fat deposition is comparable to that in humans. Avian genomes lack the hallmark gene for brown adipocytes, and this trait makes them an ideal animal model to study white adipose tissue in the absence of brown adipocytes (Abe *et al.*, 2006). Further, studies have shown traits linked to fatness in chickens are similar to those linked to predisposition for obesity and diabetes in humans (Ji *et al.*, 2012; Ji *et al.*, 2014). Including the limited number of studies that use chickens as a model, most studies that have investigated fattiness in humans have primarily focused on adult models and applications (Gonzalez-Ortiz *et al.*, 2013; Hood, 1982; Newman *et al.*, 2002). This thesis will be using young broiler chickens as a model for childhood obesity, because obesity and overweight during childhood and adolescence frequently precedes obesity during adulthood.

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CHAPTER II
FASTING RAPIDLY INCREASES FATTY ACID OXIDATION IN WHITE ADIPOSE TISSUE OF YOUNG BROILER CHICKENS

A version of this chapter will be submitted for publication by Emmanuelle Torchon and Brynn Voy to Poultry Science.

The article will be revised by Dr. Brynn Voy and Dr. Matthew Hulver of Virginia Tech, and the final draft will be submitted for publication. The primary authors will be Emmanuelle Torchon and Dr. Brynn Voy. The secondary authors will be Dr. Matthew Hulver of Virginia Tech, who provided the chickens and the fatty acid oxidation assay, and Rodney Barnett Ray, who assisted in sample collection and assays.

2.1 Abstract

Up regulating the fatty acid oxidation capacity of white adipose tissue in mice protects against diet-induced obesity and excess plasma NEFA levels. At least part of this capacity results from the induction of brown-like adipocytes within classical white depots, rendering it difficult to determine if white adipocytes contribute to the adaptation. Avian genomes lack a gene for uncoupling protein 1 and are devoid of brown adipocytes, making them a useful model in which to study lipid metabolism in white adipocytes. We recently reported that a brief (5 hr) fasting period significantly upregulated expression of genes involved in mitochondrial and peroxisomal fatty acid oxidation in white adipose tissue of young broiler chickens. The objective of this study was to determine if the effects on gene expression manifested in increased rates of fatty acid oxidation. Abdominal

adipose tissue was collected from 21 day-old broiler chicks that were fasted for 3.5, 5 or 7 hrs, or fed ad libitum (controls). Fatty acid oxidation was determined by measuring and summing $^{14}CO_2$ production and ^{14}C-labeled acid-soluble metabolites from the oxidation of [1-14C] palmitic acid. Citrate synthase activity was measured spectrophotometrically. Fasting induced a progressive increase in complete oxidation which was significantly different from controls in the 5.5 hr ($p=0.0037$) and 7 hr ($p=0.0021$) groups (1.14, 1.2, 1.49 and 1.95 nmol/mg protein/hr; control, 3.5, 5 and 7 hrs, respectively). Citrate synthase activity increased significantly but only after 7 hrs of fasting. Fasting did not significantly alter the production of acid soluble metabolites, an index of incomplete fatty acid oxidation. These results confirm that fasting rapidly increases fatty acid oxidation in white adipose tissue by upregulating the transcription of key regulatory enzymes and proteins. Identifying the underlying mechanism may provide new therapeutic targets to increase fatty acid oxidation in situ and interventions that protect against obesity and the detrimental effects of excess NEFA on adipocyte insulin sensitivity.

2.2 Introduction

Fatness is considered a waste of energy and an economic burden for the poultry industry. Over the past 70 years, broiler chickens have been genetically selected for rapid body weight gain, increased feed efficiency, and increased breast weight. Around the time of World War II, to raise a chicken to three pounds, poultry producers required more than 85 days. However, modern broiler strains selected for rapid growth (42 days for 5 pounds) carry desirable traits such as decreased rearing time and increased meat yield, compared to ancestry meat-type strains (Havenstein *et al.*, 2003). Modern commercial broilers have also increased in fatness with this shortened growth period, which is a major concern to producers and consumers as excess body fat negatively impacts productivity and fertility (Collins *et al.*, 2014). As such, various approaches have been used to address the fatness problem that intensive selection has caused.

Earlier studies in poultry have focused on the liver to combat fatness, as it has been confirmed that fat deposition in poultry is dependent on the diet and hepatic *de novo* lipogenesis (Hermier, 1997; Hermier *et al.*, 1989). Beta oxidation of fatty acids is carried out primarily in mitochondria, and in the avian and humans fatty acid synthesis predominantly occurs in the cytoplasm of hepatocytes and is limited in adipose tissue.

Using feed restriction, Richards *et al.* (2003) reported an effective reduction in the abdominal fat and a decrease in hepatic expression of lipogenic genes in broiler breeders during pre-light. An earlier study by Zhong *et al.* (1995) showed feed restriction, from

seven to 12 days of age, inhibited hepatic lipogenesis and increased the number of abdominal fat cells per gram fat tissue compared to birds fed ad lib.

Others have considered a reduction in daily caloric intake, and altering the dietary protein and protein intake to decrease fat deposition in poultry (Fouad *et al.*, 2014). Fan *et al.* (2008), using ducks from 14 to 42 days of age, reported that the relative breast and leg muscles were unchanged while adiposity decreased when the dietary energy level was reduced from 2,900 to 2,700 kcal/kg. Rabie *et al.* (1998) focused on altering the dietary energy levels of commercial broiler chickens during the growing phase, from 18 to 53 days, and reported that dietary supplementation of L-carnitine in tandem with reduced caloric intake decreased the absolute weight of the abdominal fat pad without negatively impacting growth traits.

Insulin cascades to induce glucose entry into cells and inhibit lipolysis in times of energy surplus. Lipolysis is activated in times of a low energy, such as during fasting, to provide fatty acids for energy. In broilers, the study of fatness has been focused on the liver, as it is the primary organ of lipogenesis and adipose tissue has been delegated as a dormant storage tissue (Ji *et al.*, 2012). However, using microarray and QPCR, we have discovered that a short term five hour fast increases gene expression for protein and lipid catabolism, and signaling through lipid mediators in visceral white adipose tissue of broiler chickens. Furthermore, the gene expression profiles for glucose metabolism, lipid synthesis, and adipogenesis were reduced in the white adipose tissue of fasted broilers

compared to those that were fed. The five hour feed restriction also significantly increased the expression of genes for fatty acid oxidation such as CPT-1a, and ACOX1 (Ji *et al.*, 2012). Together, our previous reports elucidated that there are pathways in white adipose tissue to locally oxidize lipids and fasting consequently revealed that the pathway can be manipulated to potentially reduce fat accumulation in commercial broiler chickens. In this study, we investigate if these gene expression profiles previously stimulated by fasting manifest as increased fatty acid oxidation and the time course in white adipose tissue.

2.3 Materials and Methods

2.3.1 Animals

Twenty-eight Cobb 500 broiler chicks were used for this study and fed standard commercial diets. The animal care protocol included humane euthanasia and was approved by the Institutional Animal Care and Use Committee (IACUC) of The University of Tennessee and Virginia Polytechnic Institute and State University. Birds were grown under standard management conditions, having free access to water and feed until 21 days of age. On day 21, a set of eight birds were fed ad-lib while the remaining were restricted from feed for three hours (n=4), five hours (n=8), and seven hours (n=8) by removing feed and water from bird cages. The birds were then euthanized by cervical

dislocation and samples of adipose tissue and liver were collected and utilized or immediately snap-frozen in liquid nitrogen and then stored at −80°C until analysis.

2.3.2 Blood serum parameters

Blood was collected from birds and transferred into 10 ml SST tubes (Fisher Scientific, Pittsburgh, PA) that were centrifuged at 2000 x g for 10 minutes then the serum was decanted from the top layer. Samples were analyzed for glucose and non-esterified free fatty acids (NEFA). Glucose levels were measured using a glucose oxidase method via a colorimetric kit (Cayman Chemical, Ann Arbor, MI). Non-esterified fatty acid levels in the serum were measured using a commercially available colorimetric assay kit (Wako Chemicals, Neuss, Germany).

2.3.3 Fatty acid oxidation assay

Four birds were randomly selected from each treatment and fatty acid oxidation was measured from freshly harvested chicken adipose tissue homogenates. Fatty acid oxidation was assessed by measuring the oxidation of 1-^{14}C palmitate (Perkin-Elmer, Waltham, MA, USA) as previously described in (Zhang *et al.*, 2014).

Approximately 200 mg of tissue sample was added into buffer (0.25 M sucrose, 1 mM EDTA, 0.01 M Tris-Cl, and 2 mM ATP, at pH 7.4) at a 1:20 (weight : volume), minced with surgical scissors, then homogenized on ice in a 2 ml glass tube at 300 RPM in 30

second pulses for 5-6 minutes. A 48-well cell culture plate (Costar, Cambridge, MA) was modified with small grooves between adjacent wells for CO_2 to diffuse freely. Aliquots were made at 40-µL volumes and were plated in quadruplets into the modified cell culture plate. One hundred sixty µl of reaction mixture (pH 7.4) started the reactions at final concentrations (in mM) of 0.2 palmitate ([1-^{14}C]palmitate at 0.5 µCi/mL), 100 sucrose, 10 Tris-HCl, 5 potassium phosphate, 80 potassium chloride, 1 magnesium chloride, 0.1 malate, 2 ATP, 1 dithiothreitol, 0.2 EDTA, 1 l-carnitine, 0.05 coenzyme A, and 0.5% fatty acid free bovine serum albumin. Plates were sealed with parafilm and a siliconized rubber gasket, incubated in a shaking 37°C water bath for 1 hour, and 100 µL 70 % perchloric acid was added to the incubation wells. Following the plate transfer to an orbital shaker, $^{14}CO_2$ was trapped in the adjoining well in 200 µL of 1 M NaOH for 1 hour. Radioactivity of was assessed with 4 mL Uniscint BD (National Diagnostics, Atlanta, GA)using liquid scintillation counting (Cortright, Sandhoff et. Al, 2006).

2.3.4 Citrate synthase activity assay

The activity of citrate synthase (CS) was measured by the reduction of 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) over time from adipose tissue of 4 birds from each treatment group. CS is an enzyme of the citric acid cycle which is rate limiting as the enzyme catalyzes the reaction between acetyl coenzyme A (acetyl CoA) and oxaloacetic acid (OAA) to form citric acid in the presence of water. When the thioester of acetyl CoA

is hydrolyzed a thiol group (CoA-SH) is formed. Available DTNB is reduced by CoA-SH and produces TNB which turns yellow.

Tissues were homogenized and diluted 1:5 in duplicates by 170 µl reaction solution (0.1 M Tris buffer at pH 8.3, 1 mM DTNB in 0.1 M Tris buffer, and 0.01 M oxaloacetate in 0.1 M Tris buffer). Thirty µl of three mM acetyl-CoA was added to initiate the reaction while absorbance was read over two minutes, then measured at 405 nm at 37 °C every 12 seconds for seven minutes using the spectrophotometer (SPECTRAmax ME, Molecular Devices Corporation, Sunnyvale, CA, USA). The maximum CS activity was calculated and reported as nmol mg^{-1} min^{-1} as previously described by Zhang *et al.* (2014).

2.3.5 Adipose tissue metabolite extraction for liquid chromatography with tandem mass spectrometry

Metabolites were extracted from 21 day old abdominal adipose tissue of 18 chickens using the solvent combination of methanol and chloroform at −20 °C. Approximately 30 to 50 mg of frozen fat was pulverized in liquid nitrogen and the pulverized tissue was collected into 1.3 ml of 100% methanol. Samples were homogenized and metabolites were extracted using chilled solvents (at −20°C) and 4°C centrifuge. A -80°C cold box was used to hold samples to avoid overheating in-between steps. Then samples were incubated for 15 minutes at −80 °C and centrifuged for 5 minutes at 4 °C at a speed of (13,200 rpm). Then supernatant was transferred to a

separate vial, and the precipitate was extracted once more following the aforementioned procedure. Then the complete samples were pooled together and 600 µl of supernatant was extracted and transferred to a 1 ml dram vial. The supernatant was dried under a stream nitrogen for approximately 30 minutes, and re-suspended in 150 µl of deuterated water. Re-dissolved samples were placed into HPLC vials then LC/MS analyses were performed using Q Exactive hybrid quadrupole-Orbitrap mass spectrometer (Thermo Scientific, USA). Each sample was run in triplicates, metabolites were separated by mass, peaks were chosen manually using Xcalibur (Thermo Fisher Scientific Inc., Waltham, MA), and data were exported into Microsoft Excel.

2.3.6 Statistical analysis

All data were analyzed for normality, homogeneity of variance using SAS (version 9.4, SAS Institute Inc., Cary, NC). Statistical significance was set to $P < 0.05$. Using a mixed-design analysis of variance (MMANOVA) for a completely random design, Tukey's Honestly Significant Difference (HSD) post hoc comparisons were determined for each data set. Significance levels are noted in figure legends and data are presented as means ± standard error. All figures and tables for this section are in the appendix at the end of this chapter.

2.4 Results

2.4.1 Serum glucose and NEFA

In the fed state, serum glucose concentrations for broiler chickens have been reported to range from 156 to 330 mg/dL (Scanes, 2008). Fasting significantly decreased serum glucose (mg/dL). As shown in panel A of Figure 2.1, five and seven hours of fasting significantly (p=0.0269) decreased glucose concentration in serum by 12.4% and 11.1%, respectively, as compared to ad lib feeding control. Three hours of fasting did not differ from the control.

Pro-longed fasting significantly increased the average NEFA levels in serum (mg/dL), as shown in panel B of Figure 2.1. Seven hours of fasting significantly (p=0.0024) increased serum NEFA levels by 123.1% relative to control. NEFA levels in serum were not significantly affected by three or five hours of fasting, but increased by 30.2% and 21.1% respectively.

2.4.2 Fatty acid oxidation and citrate synthase enzyme activity

Measuring the activity of palmitate breakdown is expressed as the rate of radio labelled carbon dioxide [$^{14}CO_2$] and acid soluble metabolites (ASM) production. ASM production represents rate of incomplete palmitate breakdown into acyl-CoA molecules of no more than 4 carbons that are acid soluble but have not entered the TCA cycle. Figure

2.2 Panel A reports the average complete oxidation of palmitate was measured using the flux of $^{14}CO_2$ (CO_2 nmol/mg of protein/hr) through the TCA cycle for ad lib feeding and three, five, and seven hours fasting respectively. Five and seven hours of fasting significantly (p=0.025) increased the rate of complete fatty acid oxidation by 55.4% and 52.5% relative to the fed control. Three hours of fasting increased fatty acid oxidation by 18.8% but was not significantly changed relative to the fed birds.

Panel B of Figure 2.2 reports the average rate of radio labelled palmitate catabolism into ASM (nmol/mg of protein/hr) for ad lib feeding and three, five, and seven hours of fasting (p=0.544). Fasting did not change the rate of incomplete palmitate breakdown.

The ratio of $^{14}CO_2$ production and ASM production represent beta oxidative efficiency due to an increase of ASM without citric acid cycle accompaniment to clear the ASM represents mitochondrial overload (Zhang *et al.*, 2014). As shown in Figure 2.2 panel C, fasting significantly (p=0.0044) increased beta oxidative efficiency after five and seven hours by 55.0% and 59.2% respectively, relative to the control. Fasting for three hours increased the ratio by 1.2%, which was not significant. Restricting chickens from feeding for seven hours significantly (p=0.0118) increased the activity of the first enzyme of the citric acid cycle, citrate synthase, by 23.1% relative to the control, as shown in panel D of Figure 2.2.

Figure 2.3 shows $^{14}CO_2$ production on the X-axis and citrate synthase enzyme activity on Y-axis. As represented by the Pearson correlation coefficient of 0.619 (p=0.0004), the graph indicates that increasing the duration of fasting significantly increased the oxidative output of the citric acid cycle in parallel to citrate synthase enzyme activity indicating a positive correlation.

2.4.3 Metabolites discovered in fasted abdominal fat

Adipose tissue metabolomics were performed to investigate the physiological responses of fasting and feeding to identify the composite effects of time dependent feed restriction on chicken adipose tissue. As seen in our previous studies, there are a number of other metabolites that differed between lean chickens, fasted chickens and fatty broilers (Ji *et al.*, 2012; Ji *et al.*, 2014). Table 1 reports the metabolites that significantly differed across treatments.

2.5 Discussion

In the present study, we examined the time dependent effects of short-term fasting on the capacity of white adipose tissue to oxidize fatty acids. The action of insulin is to regulate glucose concentrations throughout the body, however type 2 diabetes renders the regulation of glucose independent of insulin (Gannon *et al.*, 1996). In mammals, energy homeostasis is regulated through both insulin and glucagon activity.

Fasting adjusts energy metabolism in muscles from glucose utilization to fatty acid utilization, to maintain metabolic homeostasis (Randle *et al.*, 1963). However, Edwards *et al.* (1999) reported that metabolic homeostasis in chickens is predominantly regulated by glucagon.

Circulating NEFA and glycerol concentrations are elevated in obese humans, however we have previously reported in Ji *et al.* (2014) that elevated serum NEFA is a characteristic of leanness in chickens. Mechanistically, leanness in chickens must stimulate increases in adipose tissue lipase activities because of increased whole body energy demand. These may result in greater levels of fatty acids released in circulation (Ji *et al.*, 2014). Some studies in humans, chickens, and rodents report increased serum lipolysis as a characteristic response of whole body metabolism to fasting (Nielsen *et al.*, 2014; Wang *et al.*, 2003). In the fasted state, insulin status in chickens remains unchanged while circulating glucagon increases after a 24 hours (Edwards *et al.*, 1999). After 12 and 24 hour fasts, plasma glucose levels decrease and plasma NEFA levels increase (Abe *et al.*, 2006).

Our results are in agreement with previous reports that fasting causes decreased serum glucose and increased serum NEFA levels in chickens (Abe *et al.*, 2006; Edwards *et al.*, 1999). However, these results are not restricted to prolonged fasting, as our findings extend to include that fasting causes serum glucose levels in chickens to drop and serum NEFA levels to rise following short term five and seven hours fasts. Although insulin and

glucagon concentrations were not measured, our results suggest that, as represented by the inverse relationship between serum glucose and NEFA statuses, fasting altered energy homeostasis towards glucose conservation and lipid catabolism in young chickens.

Lipolysis is the crucial catabolic response to negative energy balance that causes the glucose-sparing effect explained by the Randle cycle, and provides a sufficient supply of lipid substrates for oxidative metabolism (Hue *et al.*, 2009; Nielsen *et al.*, 2014). Fasting causes negative energy balance (Fruhbeck *et al.*, 2014), but there were no observed changes in energy metabolism after a three hour fast and seven hours of fasting was required to observe all expected changes in metabolism. This suggests that energy substrates remain at an equilibrium in broiler chickens after three hours of fasting, because (i) the increased energy demand after a three hour fast is insufficient to influence serum glucose levels or lipase activities, or (ii) other regulators of energy present in broiler chickens compensate for the deficit in feed intact.

We observed that five hours of fasting was sufficient to deplete serum glucose concentrations and stimulate adipose fatty acid oxidation but was insufficient to increase lipolysis. This suggests three things: (i) the conservation of glucose, (ii) for energy, tissues are utilizing already circulating NEFA, and (iii) lipase activities remain normal. We postulate serum fatty acid concentrations possibly drop below an ambiguous threshold, preceding the spike in serum NEFA levels following a seven hour fast. Threshold signaling

stimulates lipase enzymes and activities, compensating for the energy deficit by increasing lipolysis.

Uncoupling protein 1 (UCP1) is the hallmark gene for brown adipocytes and having the capacity to oxidize fatty acids for thermogenesis is a general characteristic of brown adipose tissue. The avian genome lacks UCP1 (Mezentseva *et al.*, 2008), but the homologues of UCP1 (i.e. UCP, UCP2, and UCP3), expressed in skeletal muscle and adipose (Boss, Muzzin*, et al.*, 1998) are present. This characteristic, and their susceptibility toward rapid fat deposition due to rapid growth (Collins *et al.*, 2014), makes the modern broiler chicken an ideal model organism to study white adipose tissue.

In many organisms, lipid metabolism in skeletal muscle is very well characterized, apart from the function of the UCP1 homologues. The proposed physiological role of UCP1 homologues, although the mechanisms remain unclear, is the regulation of lipids and lipid substrates for fuel (Dulloo *et al.*, 2004). Fasting studies have provided evidence supporting this hypothesis. In chicken skeletal muscle, fasting resulted in gene expression increases of UCP1 homologues (Abe *et al.*, 2006). Samec *et al.* (1998) reported that fasting, in parallel with increased lipid utilization and glucose sparing, in rats increased UCP1 homologues gene expressions. Another study showed a positive correlation between serum free fatty acids and UCP3 mRNA expression in skeletal muscle of obese adults (Boss, Bobbioni-Harsch*, et al.*, 1998). In addition, another study reported that mRNA expressions of key lipid oxidation regulators (e.g. carnitine palmitoyl transferase

1a (CPT-1a) and medium chain acyl-coA dehydrogenase (MCAD)) increased in skeletal muscle of rats in response to fasting (Samec *et al.*, 2002).

In skeletal muscle of chickens, prolonged fasting caused up-regulated gene expression patterns of CPT-1a, the rate-limiting enzyme for mitochondrial fatty acid oxidation that transports fatty acids into the mitochondria to undergo β-oxidation (Serra *et al.*, 2013), and long-chain acyl CoA dehydrogenase (LCAD) (Abe *et al.*, 2006). The transcriptomic profile of visceral white adipose tissue after a short term five hour fast showed increased expressions of key lipid oxidation regulators (e.g. pyruvate dehydrogenase kinase 4 (PDK4) and early growth response 1 (EGR1)), and decreased expressions of genes key in glucose metabolism and fatty acid synthesis (e.g. ATP citrate lyase (ACLY) and acetyl-Coenzyme A carboxylase alpha (ACACA)) of young broiler chickens (Ji *et al.*, 2012). Our results support previous reports that fasting stimulates lipolysis, decreases circulating glucose, and switches fuel utilization towards lipid catabolism and glucose sparing. To our knowledge, no studies have reported the fatty acid oxidation capacity of broiler chicken visceral white adipose tissue following short term fasting. Citrate synthase is the rate limiting enzyme that regulates the entry of acetyl-CoA into the TCA Cycle to produce citrate in the presence of oxaloacetate. In the present study, we show that following a longer fasting time course, seven hour feed restriction, the activity of CS significantly increased in visceral white adipose tissue of broiler chickens. This heightened activity, in conjunction with increased complete palmitate oxidation,

demonstrates the capacity of white adipose tissue to oxidize fatty acids locally. Further, our findings indicate an increased beta-oxidative efficiency, and a positive correlation between citrate synthase enzyme activity and $^{14}CO_2$ production.

Measuring the effect of fasting or feeding on the rates of metabolism, although more informative, would require the use of isotopic labeling (e.g. by feeding 1-13C-labelled glucose), which was not done in this study. For the purpose of discovery, liquid chromatography coupled with mass spectrometry were used to investigate differences in metabolite pool size differences between fasted and fed energy states. We have previously reported a difference in adipose tissue metabolites of lean chickens and fasted broilers in comparison with fed broiler chickens (Ji *et al.*, 2012; Ji *et al.*, 2014). Our findings support the previous report by demonstrating the significant differences of metabolites that differed between fed and fasted chickens. We observed differences in some metabolites known to have antioxidant properties, such as ascorbate, urate, and glutathione disulfide. In particular, glutamate, oxogluterate, and tyrosine are metabolites known to be involved in oxidation that differed among fed, three, five, and seven hour fasted broiler chickens, which supports the observed changes in lipid catabolism across treatments.

Taken together, our findings provide compelling evidence that (i) white adipose tissue, devoid of brown adipocytes, is capable of engaging in elevated rates of fatty acid oxidation, (ii) there is a positive association between the efficiency of white adipocytes to

catabolize lipids locally and the length of fasting, (iii) during negative energy balance, elevated levels of NEFA are accompanied by increased lipid oxidation, and (iv) the transcriptional changes in fasted white adipose that we previously reported (Ji *et al.*, 2012) translate into increased lipid catabolism in parallel to citrate synthase enzyme activity.

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2.7 Appendix: Figures and Tables

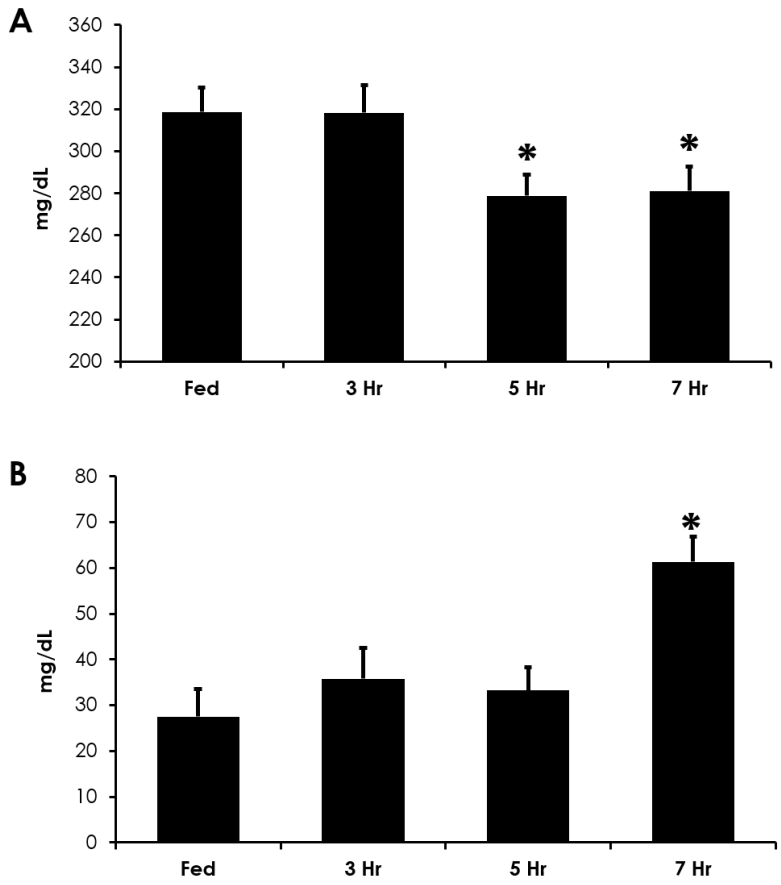
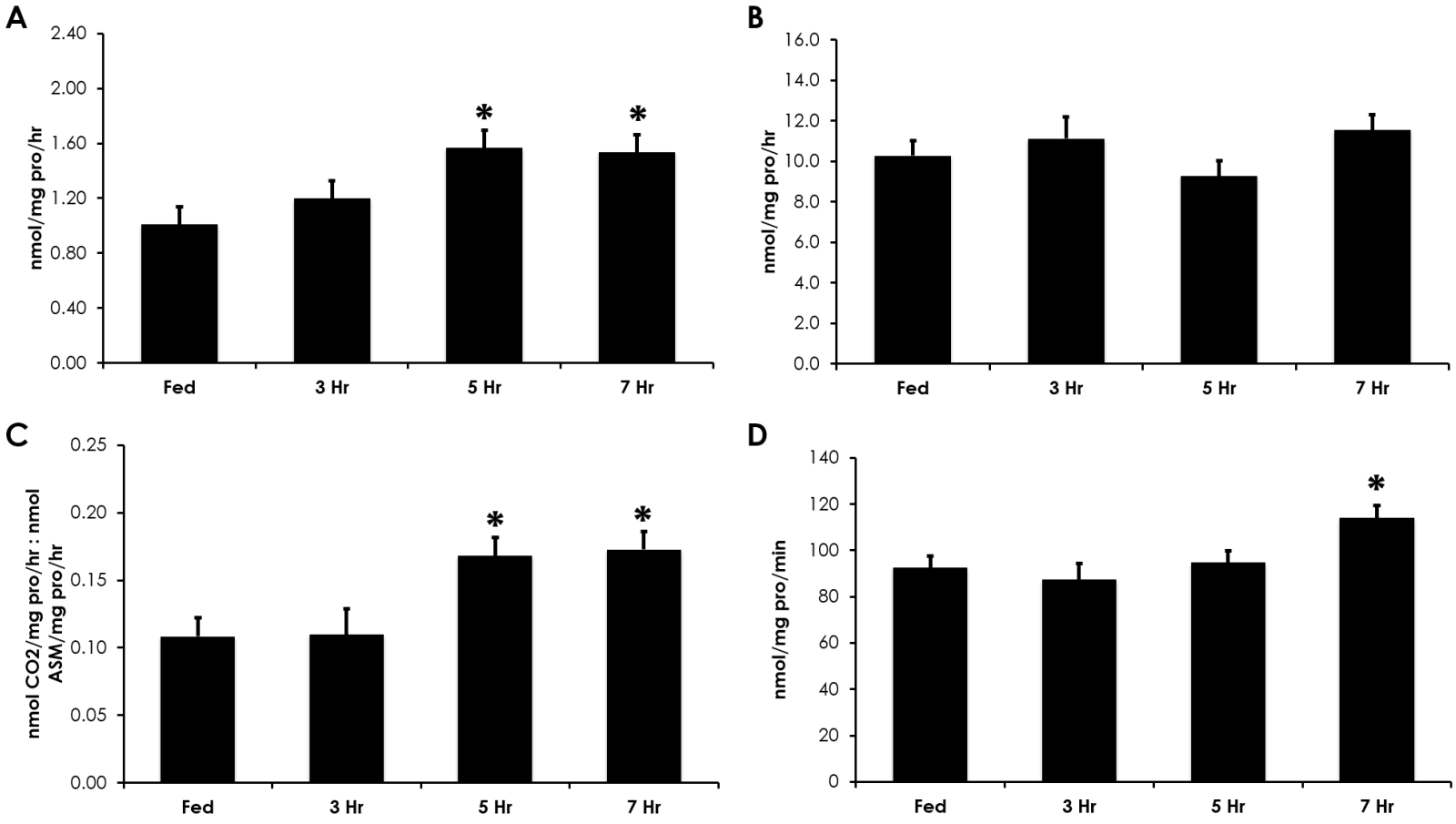


Figure 2.1 Serum glucose and free fatty acid levels

Values are group averages and error bars are SEM. Treatments: control (Fed) (n=5), fasted for three hours (3 Hr) (n=4), fasted for five hours (5 Hr) (n=7), and fasted for seven hours (7 Hr) (n=6). A. serum levels of glucose expressed in mg/dL with P=0.0269 and the y-axis starting at 200 mg/dL. B. serum NEFA levels expressed in mg/dL with P=0.0024.

Figure 2.2 Fatty acid oxidation and citrate synthase activity in abdominal fat.

Values are group averages and error bars are SEM. Treatments: control (Fed) (n=8), fasted for three hours (3 Hr) (n=4), fasted for five hours (5 Hr) (n=8), and fasted for seven hours (7 Hr) (n=8). A. 14CO2 production (nmol/mg pro/hr) with P=0.0246, B. acid soluble metabolites (^{14}ASM) (nmol/mg pro/hr) with P=0.1597, C. ratio of $^{14}CO_2$: ^{14}ASM production with P=0.0044, and D. citrate synthase enzyme activity (nmol/mg pro/min) with P=0.0118.


A
2.40
2.00
1.60
1.20
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nmol/mg pro/hr
*
*
Fed
3 Hr
5 Hr
7 Hr
B
16.0
14.0
12.0
10.0
8.0
6.0
4.0
2.0
0.0
nmol/mg pro/hr
Fed
3 Hr
5 Hr
7 Hr
C
0.25
0.20
0.15
0.10
0.05
0.00
nmol CO2/mg pro/hr : nmol ASM/mg pro/hr
*
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Fed
3 Hr
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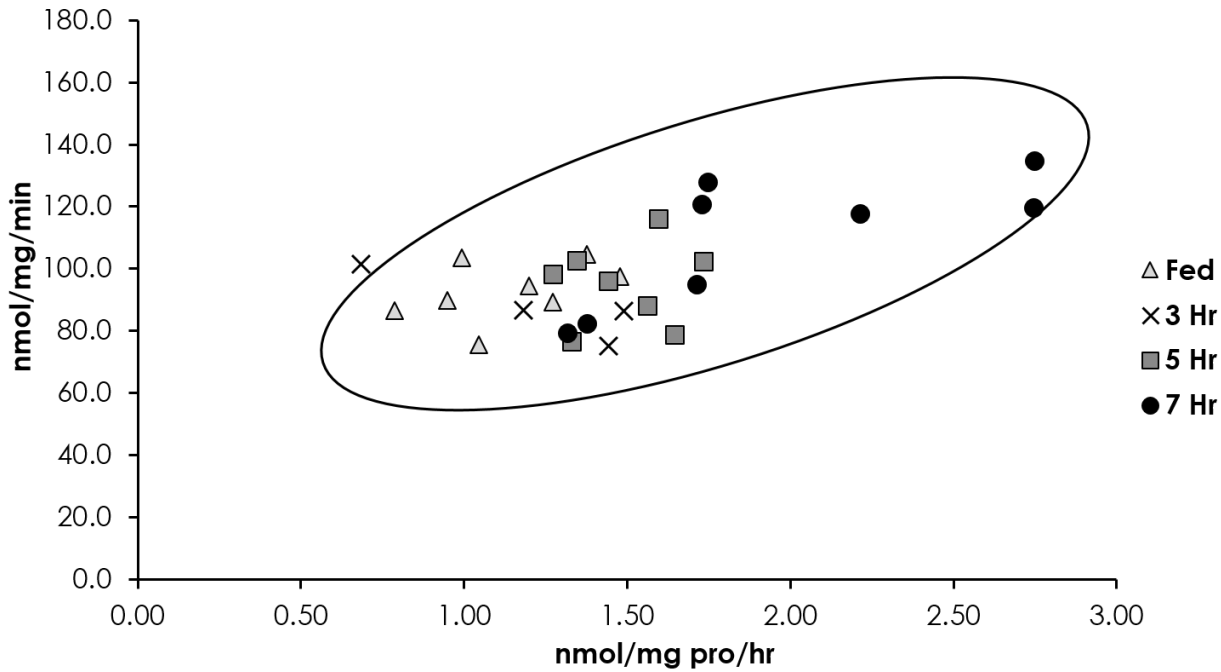

Figure 2.3 Correlation between CO_2 production and citrate synthase enzyme activity.

Oxidative output as $^{14}CO_2$ production is on the X-axis and citrate synthase enzyme activity is on the Y-axis of fasted and fed broiler chicken white adipose tissue. Treatments: control (Fed) (n=8), fasted for three hours (3 Hr) (n=4), fasted for five hours (5 Hr) (n=8), and fasted for seven hours (7 Hr) (n=8). $^{14}CO_2$ production on X-axis and citrate synthase enzyme activity on Y-axis. Pearson correlation coefficient = 0.619 with P=0.0004.

Table 2.1 Abdominal adipose tissue metabolites

Compounds	P-values
N-Acetyl-L-alanine	**0.008161**
Acetyllysine	**0.000744**
N-Acetylornithine	**0.012155**
L-Arginine	**0.038796**
Ascorbate	**0.014388**
Cellobiose	**0.000923**
CMP	**0.037222**
L-Cystine	**0.03684**
D-Glucono-1-5-lactone	**0.039805**
L-Glutamate	**0.007629**
Glutathione disulfide	**0.009138**
Guanine	**0.030518**
leucine/isoleucine	**0.017593**
L-Lysine	**0.000497**
L-Methionine	**4.13E-05**
2-Oxoglutarate	**0.032885**
Sucrose	**0.000923**
L-Tyrosine	**0.014887**
Urate	**0.001869**

Broiler chicken white adipose tissue metabolites pools altered by feeding and time dependent fasting. Treatments: control (Fed), fasted for 3 hours (3 Hr), fasted for 5 hours (5 Hr), and fasted for 7 hours (7 Hr).

CHAPTER III
ENRICHING THE DIET IN N-3 POLYUNSATURATED FATTY ACIDS REDUCES ADIPOSITY AND ADIPOCYTE SIZE IN A BROILER CHICK MODEL FOR CHILDHOOD OBESITY

A version of this chapter will be submitted for publication by Emmanuelle Torchon and Brynn Voy to Adipocytes.

The article will be revised by Dr. Brynn Voy and the final draft will be submitted for publication. The primary authors will be Emmanuelle Torchon, Suchita Das, who assisted in sample collection and assays, and Dr. Brynn Voy.

3.1 Abstract

Epidemiological studies suggest that enriching prenatal and perinatal diets in long-chain n-3 polyunsaturated fatty acids (LC n-3 PUFA) may be a tool with which to reduce adiposity, improve metabolic status and reduce the risk of childhood obesity. We used broiler chickens, which rapidly deposit adipose tissue post-hatch and have similar metabolic features with humans, to test the hypothesis that consumption of diets enriched in LC n-3 PUFA during the early post-hatch period of rapid adipose development reduces adiposity. From seven to 21 days of age, Cobb500 broiler chicks (n=10/group) were fed isocaloric diets formulated using either lard (primarily saturated), corn oil (primarily monounsaturated), flaxseed oil (PUFA, enriched in alpha linolenic acid (ALA, 18:3, n-3)), or fish oil (PUFA, enriched in eicosapentaenoic acid (EPA, 20:5, n-3) and docosahexaenoic acid (DHA, 22:6, n-3)), each at 8% by mass. Abdominal fat pad weight, but not body weight, was significantly (p=0.0327) lower in birds fed the fish oil diet

(21.3g±5.5) than those fed lard (26.7g±5.9), but did not differ in birds fed flax and canola-oil based diets relative to lard. Fish oil also significantly reduced abdominal adipocyte size compared to all other diets ($p<0.05$). Plasma non-esterified fatty acid levels, as a reflection of lipolysis, increased in birds fed fish oil diets as compared to lard diets ($p=0.0017$). Collectively, our data suggest that altering the type of fatty acids consumed during childhood may attenuate adipose deposition and adipocyte size, which could have benefits in reducing childhood obesity.

3.2 Introduction

In the United States, Europe and Australia childhood obesity, has increased in severity and frequency since the 1980s. In 2012, in the United States, 17% of children and adolescents and 35% of adults were obese (Ogden *et al.*, 2014). The rising incidence of childhood obesity is of clear concern because obesity is associated with a myriad of comorbidities including: sleep apnea, asthma, cancer, cardiovascular disease, type 2 diabetes, nonalcoholic fatty liver disease, infertility, hypertension, and orthopedic complications (Daniels *et al.*, 2005).

Broiler chickens possess attractive characteristics that make them a model organism for the study of childhood obesity and adipose biology. Modern broilers, or meat-type chickens, have been selectively bred for the past 70 years to "improve" the meat-type strains, and consequently modern chickens carry desirable traits such as: decreased rearing time, increased breast weight, rapid body weight gain, increased feed efficiency, and increased meat yield, compared to their ancestry meat-type strains (Collins *et al.*, 2014; Havenstein *et al.*, 2003). This intensive selection has produced broilers that rapidly deposit adipose tissue and are prone to obesity relative to other types of chickens (Collins *et al.*, 2014; Ji *et al.*, 2014). Like humans, de novo lipogenesis occurs primarily in the liver rather than in the adipose tissue of chickens, and locus linked to fatness in chickens are similar to those linked to human susceptibility for obesity and diabetes (Hermier, 1997; Ji *et al.*, 2012).

Furthermore, the burden of using rodents, and other lactating animals, lies on the required use of the mother to deliver nutrients after birth until weaning, but chickens can eat independently immediately post hatch. The weeks preceding chicken sexual maturation, which occurs at approximately 14 weeks of age (Hood, 1982), resemble childhood and pre-adolescent stages in humans. Broilers rapidly deposit fat in the first several weeks of life through both adipocyte hyperplasia and hypertrophy, however hypertrophy becomes the dominant mechanism for fat deposition as they mature (Cartwright, 1991; Hood, 1982).

Lipid metabolism can be regulated by dietary nutrition to reduce fatness by inhibiting fatty acid synthesis, reducing the uptake of dietary fat, and/or promoting fatty acid catabolism. Using dietary programming, chickens offer a window to manipulate and monitor adipose development to study the effects of diet on both hyperplasia and hypertrophy. Using progressive ovum dissections, we have observed that chicks hatch with subcutaneous fat and do not develop an abdominal fat depot, which resembles the visceral fat depot of the greater omentum in humans, until the days post hatch and feeding. Consequently, abdominal fat deposition in chickens is wholly controlled by dietary nutrition.

Restricting energy intake by cutting calories and increasing physical activity is the conventional approach used to promote fat loss and prevent excessive weight gain in obesity treatments and for general weight loss. However, some studies have also shown

that diet composition can be altered to impede fat accumulation and promote fat loss (Munro *et al.*, 2013). Long chain n-3 polyunsaturated fatty acids (n-3 PUFA) and n-6 polyunsaturated fatty acids (n-6 PUFA) play pivotal roles in the regulation of inflammation, neuronal health, hormonal balance, and carbohydrate and lipid metabolism. As the average American consumes more n-6 PUFA, found in cheap hydrogenated oils, versus n-3 PUFA, the dietary imbalance between these fatty acids may play important roles in the prevalence, and health problems associated with obesity (Fekete *et al.*, 2015).

The n-3 PUFA and n-6 PUFA fatty acid contents in blood and adipose tissue have been linked to obesity. In obese children, Micallef *et al.* (2009) reported that the plasma n-3 PUFA content had a negative association, and Savva *et al.* (2004) reported the contrary with the plasma content of arachidonic acid, an n-6 PUFA. Most studies that examine the beneficial effects of n-3 PUFA using animal models, including the limited number of studies that use chickens, have used adult models (Gonzalez-Ortiz *et al.*, 2013; Newman *et al.*, 2002).

The objective of this study was to examine the effects of enriching developing adipose tissue of growing broiler chickens with different dietary oils. We enriched adipose tissue through dietary supplementation and compared their effects on adipose deposition and candidate gene expression to examine if long chain n-3 PUFA enhanced fatty acid oxidation in white adipose tissue and attenuated fat deposition. In humans and

other mammals, n-3 PUFA supplementation reduced caloric intake through appetite suppression and promoted weight loss (Harden *et al.*, 2014; Munro *et al.*, 2013). Dietary supplementation with n-3 PUFA, precursors for anti-inflammatory eicosanoids, have also affected adipose mass by increasing fatty acid oxidation in other tissues and reducing inflammation by competing with n-6 PUFA, which are precursors for pro-inflammatory eicosanoids (Fekete *et al.*, 2015; Nakamura *et al.*, 2014).

3.3 Materials and Methods

3.3.1 Animals and experimental diets

Forty 1-day old mixed sex Cobb 500 broiler chicks were obtained from a commercial hatchery and raised in stacked wire cages for this study. The animal care protocol included humane euthanasia and was approved by the Institutional Animal Care and Use Committee (IACUC) of The University of Tennessee. Birds were reared under standard management conditions, having free access to water and feed until 30 days of age. On day one until day six, birds were supplied with a standard commercial starter diet. On day seven, the four treatment diets were mixed with the base diet at 8 % fat by mass using lard (LA) (Refined Lard, Lundy's, USA), canola oil (CA) (Pure Wesson 100% Natural, ConAgra Foods Inc., USA), flaxseed oil (FL) (JEDWARDS International Inc., Quincy Massachusetts), and fish oil (FO) (JEDWARDS International Inc., Quincy Massachusetts).

At day three, birds were weighed and allocated to treatment groups to establish approximately equal weights across treatments LA (n=10), CA (n=10), FL (n=10), and FO (n=10): 67.4 g, 67.8 g, 70.2 g, and 68.0 g, respectively. For 23 days, the chickens were housed in cages with five birds/cage and weighed individually twice per week until the conclusion of the study, at 30 days. A bulk base diet, sans fat, was mixed and stored at room temperature throughout this study. Treatment feed was constituted every five days and stored at 4°C in-between feedings and all birds were left to feed and drink ad-lib throughout the study. Dietary composition of the bulk base diet is shown in Table 3.1. The birds were euthanized by carbon dioxide inhalation and blood, tissue samples, final body weight, breast weight, and abdominal fat pad weights were collected at 30 days of age.

3.3.2 Blood serum parameters

A 5 ml syringe with a 25 gauge needle was used to collect blood through cardiac puncture from euthanized chickens. The blood was then transferred and inverted in SST tubes (Fisher Scientific, Pittsburgh, PA) containing then stored on ice for no more than 4 hours to allow coagulation. Samples were spun for 10 minutes at 1,000 x g then transferred and stored at -80°C. Free fatty acid (NEFA) levels in the plasma were measured using a commercially available colorimetric assay kit (Wako Chemicals, Neuss, Germany).

3.3.3 Fatty acid analysis

Abdominal fat from one randomly selected bird for each treatment group was analyzed for phospholipids (PL), neutral lipids (NL), and total lipids (TL) fatty acid compositions. Frozen adipose tissue (~34 mg) was homogenized in glass tubes with a 1:2 volume ratio of chloroform and methanol approximating 1.5 mL (2x). Homogenates were then separated into three equal volume fractions and labelled PL, TL, or NL. Then, homogenates of all fractions were diluted using 1.5 mL of chloroform and methanol at a 1:2 volume ratio, and 50 µL of 17:0 internal standard (NuChek Prep, Elysian, MN) was added. Samples were vortexed, chilled for 30 min then, to ensure phase separation did not occur, the samples were again vigorously vortexed. Saturated saline and chloroform were added to dissolve proteins, carbohydrates, and polarize the solutions prior to centrifugation (900 x g) for phase separation. The lowest phase, containing the hydrophobic layer of chloroform and lipids, was carefully extracted and dried beneath a steady stream of nitrogen.

Thin layer chromatography (TLC) plates, pre-coated with silica gel 60 (Merck, Darmstadt, Germany), were labelled and divided into three ~1-1.5 cm columns (i.e. 17:0 standard, PL, and NL) with loading areas marked 2.5 cm from the bottom using a pencil. The lipids of the PL and NL fractions were re-suspended in 25 µL of chloroform that was then slowly added along the marked lines of respective lanes (2x). Quickly and gently, the TLC plates were dried beneath the hood and placed into the TLC chamber loaded

with an 8:1 volume ratio of chloroform-methanol solvent until the solvent reached the top (~15 mins), before being dried again. Then silica was scraped into glass screw cap test tubes, one centimeter above and below the loading mark for the standard and PL columns, and above one centimeter until solvent front for the NL column, because PL remain at the loading mark while NL travel upwards with the solvent.

Boron tri-fluoride in methanol and heat were used to saponify all lipid samples into fatty acid methyl esters (FAME). Hexane was used to extract and dissolve the FAME to undergo separation by gas chromatography using a Hewlett-Packard 5880 gas chromatograph (Rochester, NY) and a DB23 capillary column (0.25 mm × 30 m) (J and W Chromatography, Folsom, OH) with hydrogen as the carrier gas. Based on the known internal standard (NuChek Prep, Elysian, MN), fatty acids were identified by retention times and fatty acid composition was calculated as a mole percentage relative to total fatty acids.

3.3.4 Adipose tissue histology

Abdominal fat and subcutaneous fat, from the thigh, were removed and submerged in chilled 4% paraformaldehyde in 0.1 molar sodium phosphate buffer at pH = 7.4 for tissue fixation. Tubes were then transferred to 4°C for 12 hour incubation, washed in sodium phosphate buffer at 0.1 molar, then transferred into chilled sodium phosphate buffer for storage. Under standard tissue processing protocols, a histochemist

at Ridge Microtome (Ridge Microtome, Knoxville, TN 37932) dehydrated the samples in gradually more potent alcohol baths, removed the alcohol with a hydrophobic clearing agent, and infiltrated the tissues with molten wax. Then tissues were sliced in a microtome and mounted onto a glass microscope slide prior to light microscopical staining. Two slides were made for each sample.

Images were captured from the slide with the least defects and blood vessels with the Advanced Microscopy Group (AMG) EVOS XL Core microscope (Fisher Scientific, Pittsburgh, PA). Four images were captured on each slide under 20x magnification. Using Image J (Version 1.48, National Institutes of Health) with MRI Adipocyte tools, adipocytes were counted and area was determined using microscope settings of 3.2 um/pixel and treated with the area restriction that measurements must exceed 100 μm^2. Then, assuming circularity, adipocyte volume was calculated using the following formula = $(4/3) * \pi * (\text{Adipocyte Area } (um^2) / \pi)^{3/2}$.

3.3.5 Real Time Quantitative PCR assay

Total RNA was isolated from chicken abdominal adipose tissue collected and snap frozen from 24 mixed sex birds, with 6 birds from each treatment group. The birds were chosen based on average adiposity for each treatment ± one standard deviation. Approximately 200mg of adipose tissue was homogenized in 1 ml of TRIzol reagent (Ambion RNA, Life Technologies Corporation, Carlsbad, CA). Chloroform at 200 µl volume

was added for phase separation and samples incubated at room temperature for 3 minutes then centrifuged at 12,000 x g for 15 minutes at 4°C. The clear upper aqueous phase was transferred into fresh tubes and RNA was precipitated using 500 ul of 100% isopropyl alcohol and incubated at room temperature for 10 minutes, then centrifuged at 12,000 x g for 10 minutes at 4°C. The supernatant was removed and the RNA pellet washed with 1 ml of 75% ethanol twice and centrifuged at 7,500 x g for 5 minutes at 4°C. Then the supernatant was removed and the pellets were air dried. RNA was then re-suspended in 50 ul of nuclease free water in a 37°C water bath for 10 minutes. The quality and concentration of RNA were determined by measuring the absorbance at 260 and 280 nm on an Amersham Biosciences UltraSpec 3100 pro spectrophotometer (Piscataway, NJ). Then RNA integrity was confirmed by RNA gel electrophoresis.

CDNA was synthesized from 500 ng total RNA in 20 µL reactions using iScript cDNA Synthesis kit (BIORAD, USA), following the manufacturer's instructions with the following thermo-cycler conditions: 25°C for 5 minutes, 42°C for 30 minutes, and 85°C for 5 minutes. Primers for real time quantitative PCR (RT-QPCR) were designed by Qiagen (Venlo, Limburg, Netherlands) and integrated DNA technologies (IDT). RT-QPCR was performed in triplicates in 10µL volume reactions that contained 0.5 µL 10 fold diluted cDNA, 0.5 ul of each forward and reverse primers, and 5 ul of IQ SYBR Green Supermix Master Mix (BIORAD, USA) and 3.5 µL of water. PCR was performed for 40 cycles under the following conditions: 95°C for 1 minute, 54°C for 45 seconds, then 72°C for 2 minutes.

The housekeeping gene used for relative expression calculations was TBC1 domain family 8 [UniGene ID 1180728—Gga.10877]. Primer sequences are shown in Table 3.2.

3.3.6 Statistical analysis

Data were analyzed for normality and homogeneity of variance using SAS (version 9.4, SAS Institute Inc., Cary, NC) and statistical significance was set to $P < 0.05$. A mixed model analysis of variance (MMANOVA) using Fischer's least significant difference (LSD) post hoc comparisons for mean separation was used for each data set. Significance levels are noted in figure legends and data are presented as means ± standard error. All figures and tables for this section are in the appendix at the end of this chapter.

3.4 Results

3.4.1 Effects of dietary fatty acid supplementation on tissue fatty acid composition

The experimental diets were formulated identically except in terms of the fat source (Table 3.1), therefore observed differences in all data are credited to fat source. The effects of diet on adipose tissue phospholipid fatty acid profiles are shown in Figure 3.1. The fatty acid profile of all abdominal white adipose tissue fractions (i.e. PL, NL, and TL) from 1 randomly selected chicken for each treatment are shown in Table 3.3. As expected each fraction was enriched in the corresponding dietary fatty acids. The

membrane phospholipids of the LA group are enriched with n-6 PUFA and have the greatest n-6 : n-3 ratio (14.29) among all other groups (CA=5.22, FL=0.58, and FO=0.04), and phospholipids of the CA group show the greatest dietary MUFA (41.30%) enrichment. The FO group, being fed the only diet having very long chain n-3 PUFA, show EPA (20:5n-3) (28.69%) and DHA (22:6n-3) (16.68%) enrichments, and the FL group shows the greatest ALA (18:3n-3) enrichment (21.12%) compared to all other groups (LA=2.15%, CA=4.27%, and FO=3.97%).

3.4.2 Effects on body composition and growth rate

There were no effects observed in the chickens other than the effects of the diet among all groups. The physical effects in response to 21 day fatty acid dietary supplementation during the growth phase until day 30 are shown in Table 4. There was a difference in average body weight (p=0.0150), only in response to the LA (1752.0 ± 44.3 g) and FO (1526.0 ± 42.7 g) diets (CA=1650.0 ± 51.8 g, and FL=1694.0 ± 42.8 g). There was a diet effect on the average carcass weight, calculated by subtracting breast weight from overall body weight (p=0.039). The average weight of the whole breast muscle plate was significantly affected by dietary fatty acid composition (p=0.0054) as indicated by the FO group (309.17 ± 14.2 g) having the lightest average breast muscle weight to the FL (351.28 ± 12.9 g) and LA (388.38 ± 13.1 g) groups, but not the CA group (336.56 ± 15.7 g). The LA group showed the greatest ratio of breast weight relative to overall body weight

(p=0.048) and carcass weight (p=0.042) compared to all other groups. There was no effect of diet on the average overall body growth rate from day 7 to day 30, the omental fat pad weight, nor the percentage of omental fat tissue relative to body weight. Indicating that dietary fatty acids did not inhibit or accelerate broiler chicken growth or abdominal fat deposition.

3.4.3 Effects on Lipolysis and Adipose Tissue Cellularity

The effects of dietary fatty acid supplementation on adipocyte size and serum NEFA are presented in Figure 3.2. The averages and statistical differences among adipocytes of ≥100 μm^2, and the average adipocyte sizes for FO, FL, LA, and CA groups were calculated (n=5, 5, 5, and 4 birds, respectively). Dietary n-3 PUFA supplementation caused the average abdominal adipocyte size to decrease as shown in panel A. The lowest overall average adipocyte size, at significance level $p<0.0001$, is a result of providing dietary fat as FO (223.05 ± 6.61 μm^2). Providing dietary fat as FL (246.6 ± 6.61 μm^2) causes smaller fat cells as compared to LA (275.18 ± 6.61 μm^2) and CA (275.41 ± 7.39 μm^2) diets but not the FO diet, indicating that the length of dietary n-3 PUFA and not simply n-3 PUFA affect adipocyte cellularity.

The effect of dietary fatty acid supplementation on the average serum NEFA levels are shown in Panel B of Figure 3.2. The FO birds had the highest level of lipolysis measured from sera (10.04 ± 0.88 mg/dL) using n=8 birds at significance level p=0.002. The serum

NEFA was not influenced by the LA (5.63 ± 0.88 mg/dL), CA (6.25 ± 0.94 mg/dL), or FL (5.01 ± 0.88 mg/dL) diets, measured using n=8, 7, 8 birds, respectively.

Adipocyte volume was calculated from adipocyte size and is presented in Figure 3.3. Expressed as relative frequency percentages, FO birds, having the smallest average adipocyte size, had the highest incidence of smaller adipocyte volumes (49.2 ± 3.13 %) as compared to LA (36.0 ± 3.13 %), CA (37.7 ± 3.5 %), and FL (41.8 ± 3.13 %) of the adipocytes being less than or equal to 2000 μm^3. As cell volume increases, the incidence of larger adipocyte volumes occur as a result of LA and CA dietary supplementation. Summing the frequencies of adipocytes of 6000 μm^3 and greater yields values of 33.6%, 33.8%, 25.3%, and 18.2% for LA, CA, FL, and FO birds, respectively, indicating that providing dietary fat as LA and CA causes fat cell growth. To provide visual aid for comparison to show the effects of LA (Panel A), CA (Panel B), FL (Panel C), and FO (Panel D) on dietary fats on adipocyte cellularity, Figure 3.4 shows the histological sections of one randomly chosen abdominal fat tissue slide for each diet with 50 µm scale bars.

3.4.4. Effects on Relative mRNA Expression in Visceral White Adipose Tissue

The average mRNA expression in visceral white adipose tissue are compared across diets and relative to TBC1D8. Panel A of Figure 3.5 indicates that in broilers that consumed the lard diet, mRNA expression levels of PPARG and EGR1 were significantly elevated. Peroxisome proliferator-activated receptor gamma (p=0.0198) and EGR1

(p=0.0485) show similar expression patterns, with the canola oil and fish oil diets produced the lowest relative mRNA expressions while the flaxseed oil diet did not significantly change mRNA expressions. These two genes were chosen to investigate if the present dietary fatty acids function as ligands to regulate energy metabolism by altering gene transcription in white adipose tissue. Both PPARG and EGR1 help to regulate cell differentiation, transcription, and glucose-fatty acid metabolism.

Panel B of Figure 3.5 indicates if the present dietary fatty acids effect energy homeostasis through lipid hydrolysis and synthesis. The mRNA expression levels of lipoprotein lipase (LPL), patatin-like phospholipase 8 (PNPLA8), and phosphatidate phosphatase 1 (LPIN1) are reported. Broilers fed the lard diet showed significant elevations of PNPLA8 (p=0.0011), and LPL and LPIN1 indicate no change across dietary treatment groups.

Panel C investigates the effects of the present dietary fatty acids on expression profiles of regulatory genes for energy balance through lipid breakdown and synthesis. The mRNA expression profiles of ACOX1, PDK4 (multiplied by factor 10 to fit onto the graph), CPT-1a, and fatty acid synthase (FASN) are presented. The mRNA expression of PDK4 (p=0.0229) was highest in white adipose tissue of broilers that consumed the fish oil diet. The canola oil and flaxseed oil diets showed the lowest mRNA levels and the lard diet indicate no difference compared to other diets. The graphs of ACOX1, CPT-1a, and FASN show that there is no significant effect of dietary fatty acids on mRNA expressions.

Panel D reports mRNA expressions of the key enzyme for gluconeogenesis, phosphoenolpyruvate carboxy-kinase 1 (PCK1), and inflammatory lymphocytes, chemokine C-C ligand 20 (CCL20), are not significantly altered in white adipose tissue across in response to dietary fatty acids.

3.5 Discussion

This study investigated the effects of ad libitum feeding of diets enriched in saturated and n-6 poly-unsaturated (lard fat), mono-unsaturated (canola oil), and different n-3 poly-unsaturated (flaxseed oil and fish oil) fatty acids on fat deposition and body composition traits during the critical developmental growth period of broiler chickens, as a model for childhood obesity. Compared to dietary SFA, some studies using chicken models have reported that dietary n-6 PUFA, provided as sunflower or soybean oils, caused a reduction in fatness by decreasing adiposity, abdominal fat pads, and adipocyte sizes (Newman *et al.*, 2002; Sanz *et al.*, 2000; Wongsuthavas *et al.*, 2008). Other studies using chickens as a model have reported that dietary n-3 PUFA, provided as a mix of fish and flaxseed oils, also reduce fatness compared to dietary SFA (Gonzalez-Ortiz *et al.*, 2013; Villaverde *et al.*, 2006). Our findings support previous reports that unsaturated fatty acids cause a decreasing effect on fatness in broiler chickens. However, as the LA and CA groups had a propensity toward larger adipocytes compared to the FO and FL

groups, our findings extend to include that the type and level of fatty acid unsaturation alters fatness in broiler chickens. Further, the types of n-3 PUFAs influence glucose and lipid metabolism differently in young broiler chickens, as indicated by the FO and FL diets having conflicting effects on body and breast weights, although both diets are rich in n-3 PUFAs.

Modern meat-type chickens have been bred to yield disproportionately larger breast muscles compared to other body parts (Collins *et al.*, 2014). Carcass weight was calculated to safeguard our findings that the diet treatments truly affected whole body growth. Carcass weight was also significantly different between diet groups, supporting that differences in body weight were truly effected by dietary fatty acids and not a result of the massive breast plates.

In mammals, insulin inhibits lipolysis, and insulin sensitivity is inversely associated with the level of serum free fatty acids (Unger, 2003). However, in chickens, elevated serum free fatty acids are a characteristic of leanness (Ji *et al.*, 2014). The FO diet caused elevated levels of serum lipolysis, and the highest incidence of smaller adipocytes. Further, dietary LA caused decreased levels of serum NEFA compared to FO, and effected adipose cellularity in both subcutaneous (not reported) and visceral depots by influencing cell size towards larger adipocytes, compared to dietary FL and FO. The mechanism by which longer chained n-3 PUFAs, EPA and DHA, impact glucose and lipid homeostasis may

be through inhibition of lipid storage signals and stimulation of fatty acid catabolism, as indicated by elevated levels of circulating NEFA.

The major regulator of adipogenesis is PPARG (Rosen *et al.*, 2006) while EGR1 functions as a transcription regulator for cell differentiation and suppresses lipolysis by inhibiting adipose tissue lipase expression (Chakrabarti *et al.*, 2013). In vitro and in vivo studies in humans and rodents have demonstrated that n-6 PUFA and n-3 PUFA have contrasting effects on fatness. Some studies report that n-6 PUFA promote adipogenesis through the activity of prostacyclin (Gaillard *et al.*, 1989; Massiera *et al.*, 2003) and causes increased expression of lipogenic genes while n-3 PUFA encourage the opposite (Muhlhausler *et al.*, 2010). Although we did not measure prostacyclin, the effect of n-6 PUFA compared to n-3 PUFA is cross-species and includes chickens. This is indicated by our in vivo studies of the response of chicken adipose tissue to dietary n-6 PUFA and n-3 PUFA. The abdominal white adipose tissues of birds fed the lard supplemented diet, enriched in n-6 PUFA and SFA, had the highest mRNA expressions of EGR1 and PPARG, indicative of cell differentiation and adipogenesis. Our findings support those of Royan et. al. who reported an elevated relative PPARG expression in adipose tissue of chickens fed a diet enriched in palm oil compared to diets enriched in soybean oil, fish oil, and conjugated linoleic acid. They reported a the fatty acid composition of the dietary palm oil as having the highest fatty acid composition of SFA relative to the other dietary fats, and a n-6 : n-3 ratio of 18.31 (Royan *et al.*, 2011). Further, compared to the FO

supplemented birds, LA diet had decreased levels of lipolysis and a higher average abdominal adipocyte size, supporting and explaining the relatively heightened expression of EGR1.

Spalding *et al.* (2008) reported that adipocyte number in humans is established in early development, which reinforces the need for childhood obesity research. An isoform of PPARG that regulates adipocyte size by promoting lipid storage to prevent lipotoxicity is PPARG-2 (Medina-Gomez *et al.*, 2007). If adipocyte number is set in early childhood for chickens, we postulate that the significantly elevated PPARG mRNA expression in fat tissue of the LA broilers may be due to the presence and expression of PPARG-2 because the average abdominal adipocyte number (not reported) did not differ between diets. This may be as result of an already established adipocyte number from hatch until day 6, as chickens began their diets when aged seven days.

The patatin-like phospholipase family members have high cleavage specificity for both saturated and mono-unsaturated fatty acids (Yan *et al.*, 2005). The phospholipid fatty acid analysis reveals the relatively heightened level of MUFA in the CA and FL diets. We expected the gene expression profiles of the CA and FL diets for PNPLA8 to increase but they did not. Instead, the LA diet showed significantly increased PNPLA8 mRNA expression. Although we did not measure the positional abundance of phospholipids, we postulate that there is a greater presence of SFA, because of its straight chain structure, compared to MUFAs on the sn-1 and sn-2 positions of phospholipids.

Pyruvate dehydrogenase kinase 4 contributes to the energy metabolism by regulating energy substrates, and shifting substrate oxidation from glucose to fatty acids and stimulating glucose storage (Connaughton *et al.*, 2010; Ji *et al.*, 2012). As expected, the relative expression of PDK4 was elevated in the adipose tissue of birds fed FO compared to birds fed FL and CA diets, which had the overall lowest relative mRNA expressions for PDK4. This increased PDK4 expression in the FO diet suggests locally oxidized fatty acids in white adipose tissue, despite not having observed any changes in CPT-1a and ACOX1 expressions. Surprisingly, visceral white adipose tissue of birds fed LA diet demonstrated PDK4 expression that was not relatively different from any diet, and further investigations are necessary. The LA diet shows the highest phospholipid fatty acid mole percentage of n-6 PUFA and the FO diet shows the highest phospholipid composition of n-3 PUFA. Ferrer-Martinez *et al.* (2006) reported that a high fat diet vs a high sucrose diet did not alter the expression of PDK4 in skeletal muscle of rats. If the type of endogenous fatty acids, rather than a high fat diet, effect PDK4 expression this could offer clarification as to why the LA diet, rich in n-6 PUFA and SFA, did not have the lowest PDK4 expression.

In this study, n-3 PUFAs altered several components of adipose metabolism, however further studies are necessary to identify the underlying mechanisms through which dietary n-3 PUFAs reduce fatness. This study validates our ability to use broiler chicks as a model for human obesity research, to manipulate adipose development, and

to study the effects of nutritive changes on white adipose tissue. Enriching the diet of growing chicks in fish oil significantly reduced adipocyte size, and both flaxseed and fish oil supplementation promoted the incidence of smaller adipocytes relative to lard and canola oil supplemented diets. Dietary fatty acids had diverse effects on gene expression profiles involved in adipogenesis, insulin sensitivity, lipolysis, and gluconeogenesis. Our findings do not include evidence that dietary n-3 PUFAs acted to reduce fatness by stimulating white adipose tissue to oxidize fatty acids locally. Nevertheless, we observed that FO birds had elevated PDK4 mRNA expressions suggesting local fatty acid oxidation because PDK4 functions as a fuel switch for lipids.

Overall, diet and lifestyle are the major factors that contribute to the incidence of obesity, which presents the need for studies of dietary supplementation. This study confirms that dietary programming during development effects fat storage and adipocyte size, and additional investigations of dietary nutrition can help identify the mechanisms that network to regulate energy homeostasis as influenced by dietary programming. Collectively, our data suggest that altering the type of fatty acids consumed during childhood moderates fat deposition, and offers a preventative approach to reduce the incidence of obese children and pre-adolescents who would likely develop into obese adults.

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3.7 Appendix: Figures and Tables

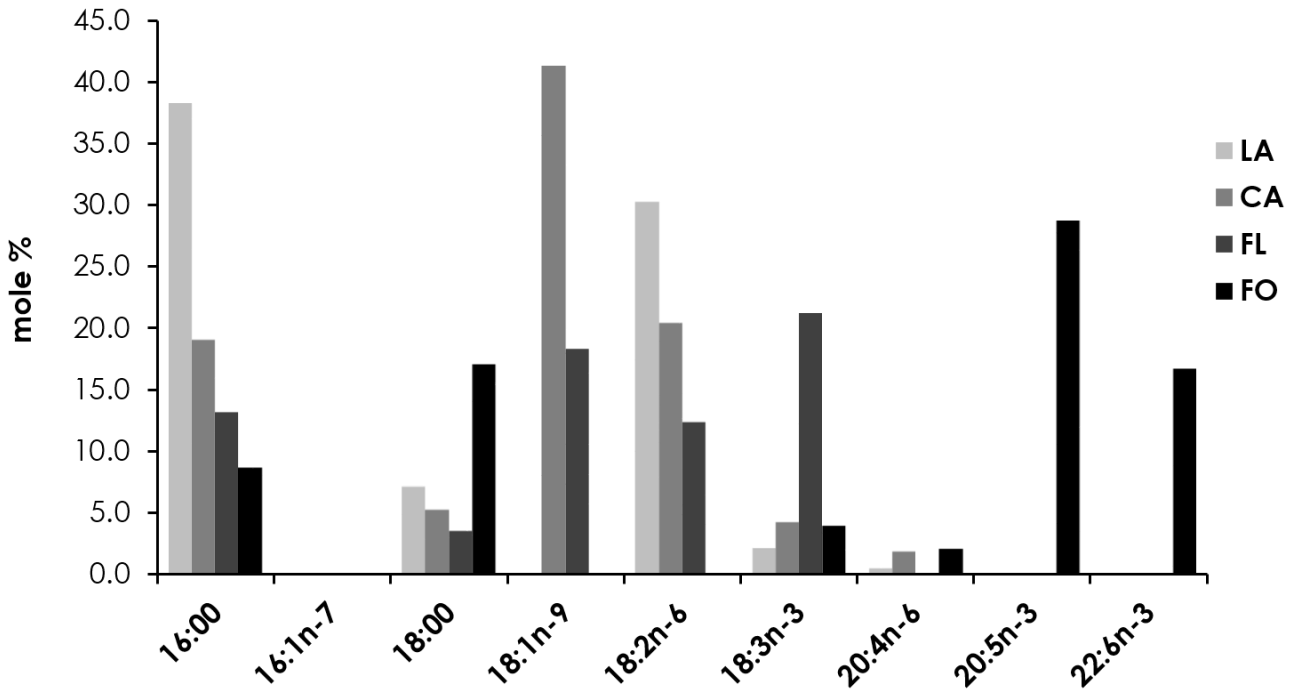


Figure 3.1 Abdominal adipocyte membrane fatty acid composition

Phospholipid fatty acid composition of visceral white adipose from 30 day old broiler chicken adipocytes expressed in mole%.

Treatment groups: 8% lard fat (LA), 8% canola oil (CA), 8% flaxseed oil (FL), 8% fish oil (FO)

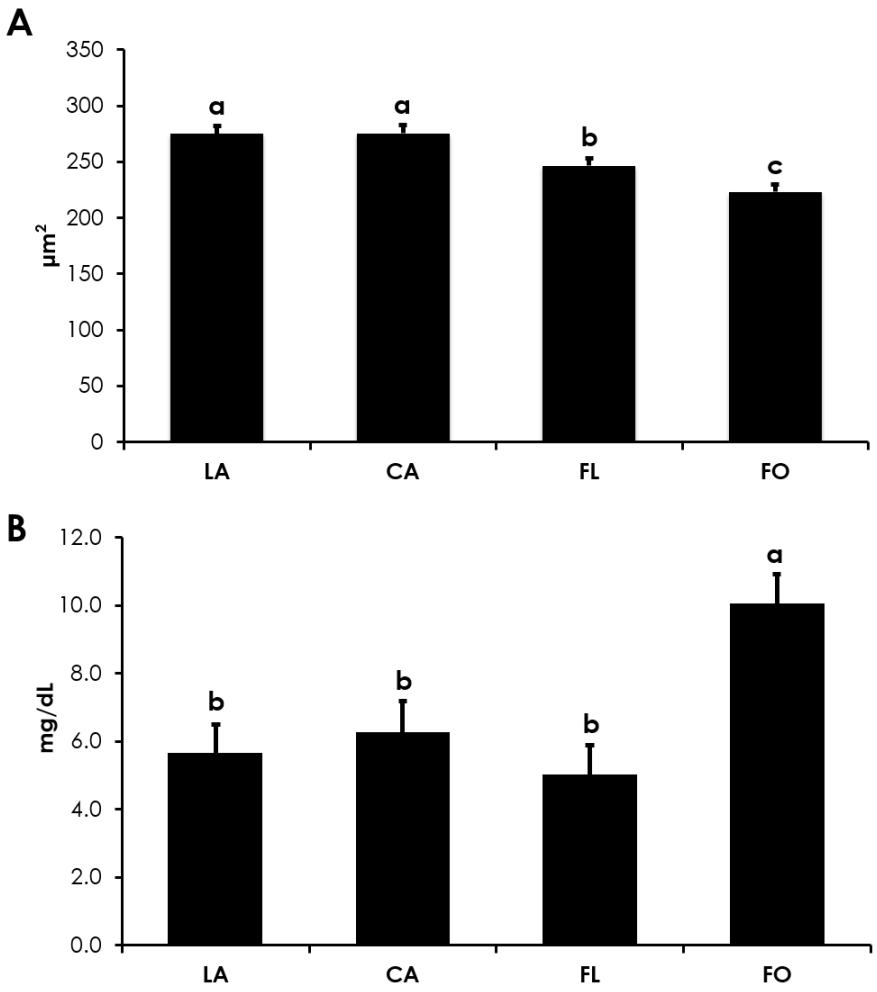
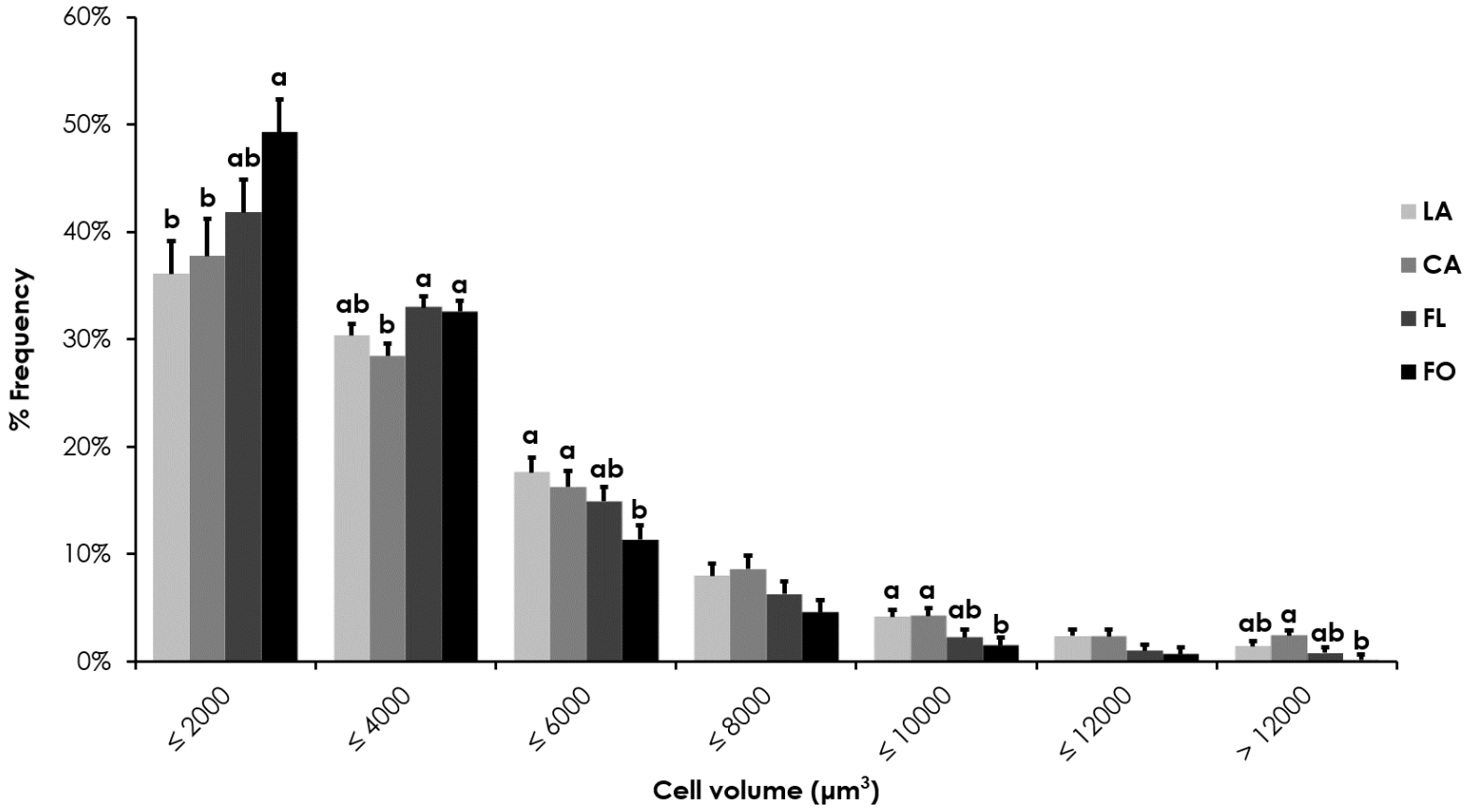


Figure 3.2 Abdominal adipocyte size and serum free fatty acid levels

Values are group averages and error bars are SEM. Treatment groups: 8% lard fat (LA), 8% canola oil (CA), 8% flaxseed oil (FL), 8% fish oil (FO). A. adipocyte area at $P<0.0001$ for LA (n=5 birds), CA (n=4), FL (n=5), FO (n=5) expressed in μm^3. B. serum NEFA levels at $P=0.0017$ for LA (n=10 birds), CA (n=10), FL (n=10), FO (n=10) expressed in mg/dL.

Figure 3.3 Relative frequency of fat cell volume in abdominal adipose tissue

Relative adipocyte volume frequency from broiler chicken white adipose tissue. Error bars are SEM. Treatment groups: 8% lard fat (LA), 8% canola oil (CA), 8% flaxseed oil (FL), 8% fish oil (FO). ≤ 2000 μm^3 (P=0.0393), ≤ 4000 μm^3 (P=0.0418), ≤ 6000 μm^3 (P=0.0324), ≤ 8000 μm^3 (P=0.0972), ≤ 10000 μm^3 (P=0.0341), ≤ 12000 μm^3 (P=0.1726), > 12000 μm^3 (P=0.0455).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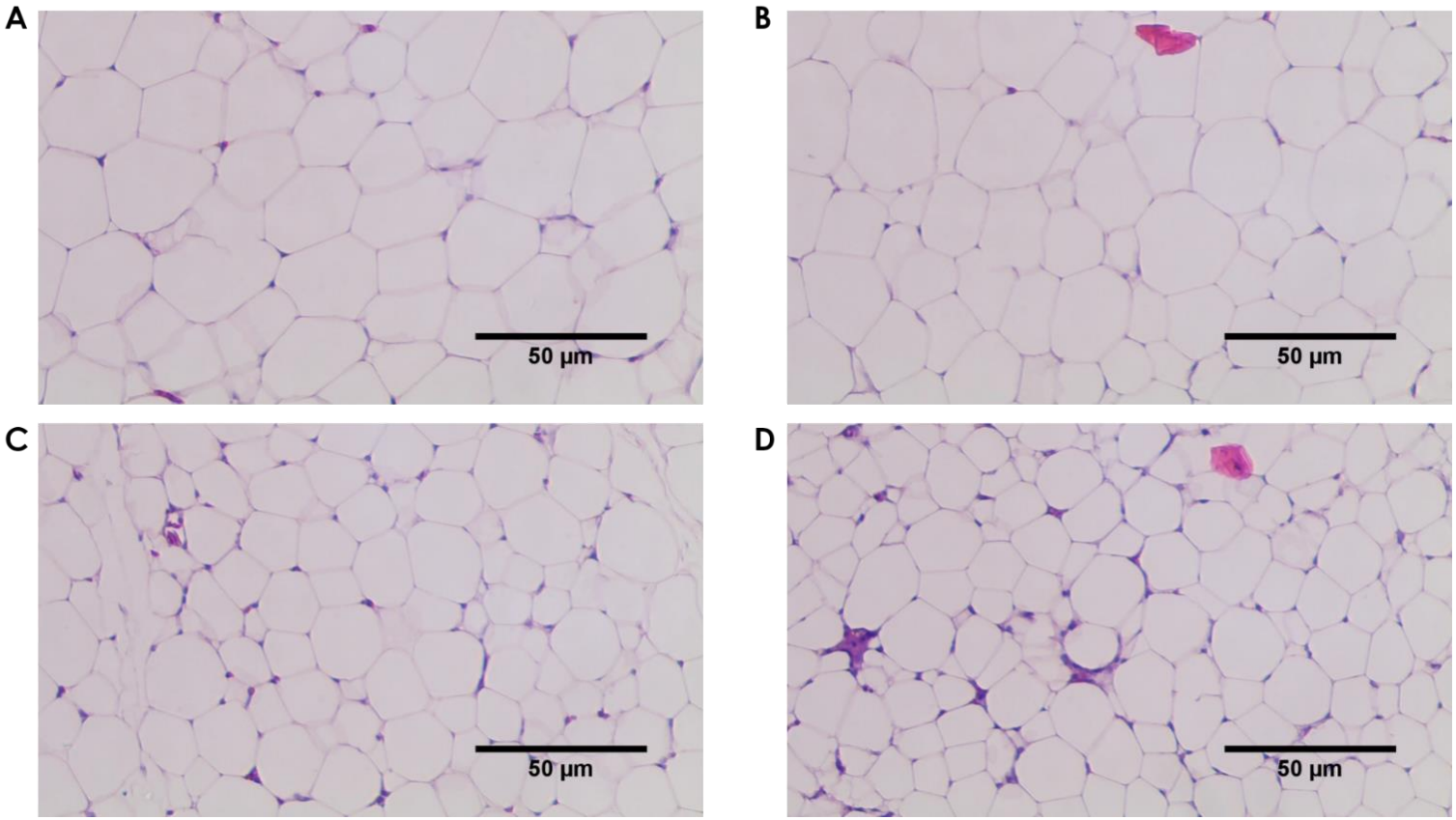

Figure 3.4 Histological cellularity of abdominal adipose tissue

Histological sections of abdominal white adipose tissue of broiler chickens. Treatment groups: A. 8% lard fat (LA), B. 8% canola oil (CA), C. 8% flaxseed oil (FL), D. 8% fish oil (FO).

Figure 3.5 Relative mRNA expression in abdominal adipose tissue.

Relative mRNA expression in abdominal white adipose tissue of young broiler chickens. N=6 birds for each diet. Values are group averages and error bars are SEM. Treatment groups: 8% lard fat (LA), 8% canola oil (CA), 8% flaxseed oil (FL), 8% fish oil (FO). A. PPARG (values divided by 10) (P=0.0198) and EGR1 (P=0.0485), B. LPL (values divided by 100) and PNPLA8 (P=0.0011) and LPIN1, C. ACOX1 and CPT-1a (values multiplied by 10) and PDK4 (values multiplied by 10) (P=0.0229) and FASN, D. CCL20 and PCK1.

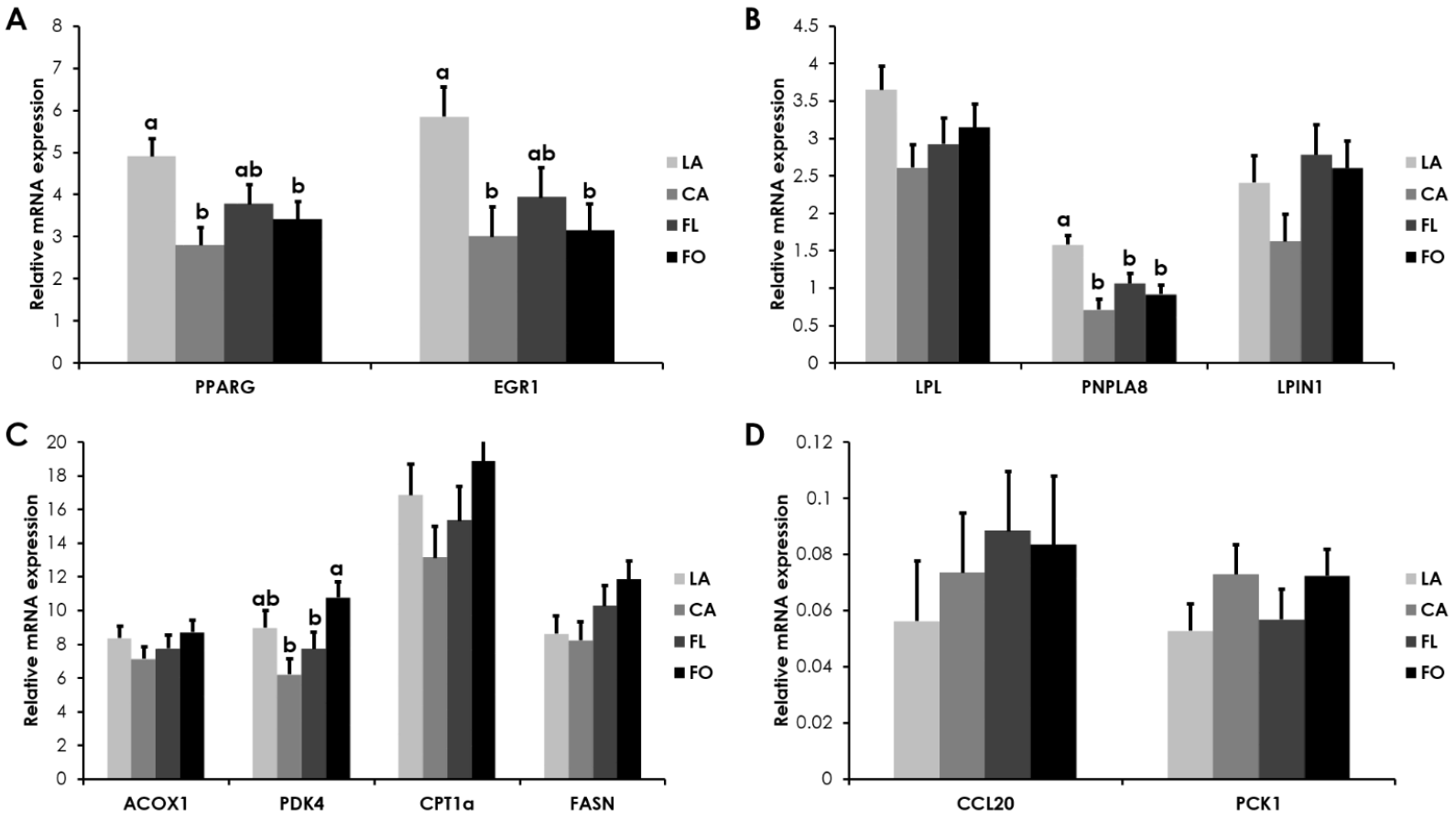
A
Relative mRNA expression
PPARG
EGR1
LA
CA
FL
FO
B
LPL
PNPLA8
LPIN1
C
ACOX1
PDK4
CPT1a
FASN
D
CCL20
PCK1

Table 3.1 Base experimental diet composition

Element	Grams (g)	Composition (%)
Corn, grain	25632.5	56.5%
Soybean meal	14424.2	31.8%
Corn gluten meal	-	-
Fish meal	-	-
Rice Bran	-	-
Vitamin Premix	226.8	0.5%
Filler	-	-
Choline, Mg/kg	90.7	0.2%
Betaine, Mg/kg	-	-
DL Met	45.4	0.1%
Salt	136.1	0.3%
Limestone	526.2	1.2%
Dicalcium phosphate	648.6	1.4%
Trace Min. Premix	-	-
Fat, animal	3628.7	8.0%
Coban	-	-
Lysine	-	-

Base experimental diet composition with values represented as the percentage by weight of dietary compound.

Table 3.2 Fatty acid composition (mole %) of abdominal fat

Fatty acid composition of abdominal fat in mole %. Snap frozen abdominal fat from one chicken was randomly selected from each diet to run the analyses.

Total FA = ∑SFA + ∑MUFA + ∑PUFA

Total SFA = C14:0 + C15:0 + C16:0 + C17:0 + C18:0 + C20:0 + C22:0 + C24:0

Total MUFA = C16:1 + C17:1 + C18:1 n-7 + C18:1 n-9 + C20:1 n-9 + C22:1 n-9

Total PUFA = C18:2 n-6 + C18:3 n-3 + C18:3 n-6 + C20:4 n-6 + C20:5 n-3 + C22:6 n-3

Total n-6 = C18:2 n-6 + C18:3 n-6 + C20:4 n-6

Total n-3 = C18:3 n-3 + C20:5 n-3 + C22:6 n-3

	Phospholipids lipids (mole%)				Neutral lipids (mole%)				Total lipids (mole%)			
	Treatments				Treatments				Treatments			
	LA	CA	FL	FO	LA	CA	FL	FO	LA	CA	FL	FO
C14:0	2.46	1.19	1.26	19.30	1.82	-	1.05	-	-	-	-	8.45
C16:0	38.26	19.05	13.10	8.64	26.03	20.94	25.24	28.35	26.36	19.47	18.87	-
C16:1n-7	-	-	-	-	8.42	6.26	8.37	9.43	7.49	4.51	5.62	12.64
C18:0	7.13	5.25	3.48	17.06	3.55	3.26	4.31	-	4.75	4.70	4.40	9.05
C18:1n-9	-	41.30	18.27	-	36.44	43.58		25.27	39.63	48.05	28.00	33.75
C18:2n-6	30.24	20.45	12.35	-	22.06	20.87	22.66	10.56	4.43	18.83	16.51	14.48
C18:3n-3	2.15	4.27	21.12	3.97	1.69	5.36	38.37	-	-	4.44	26.59	-
C20:4n-6	0.48	1.82	-	2.08	-	-	-	-	-	-	-	-
C20:5n-3	-	-	-	28.69	-	-	-	7.98	-	-	-	13.44
C22:6n-3	-	-	-	16.68	-	-	-	-	-	-	-	8.18
Total FA	80.72	93.33	69.58	96.42	100.0	100.2	100.0	81.59	82.67	100.0	100.0	100.0
Total SFA	**47.85**	25.49	17.84	45.00	**31.40**	24.19	30.60	28.35	**31.11**	24.17	23.27	17.50
Total	0.00	**41.30**	18.27	0.00	44.86	**49.84**	8.37	34.70	47.13	**52.55**	33.62	46.40
Total	32.86	26.54	33.47	**51.42**	23.75	26.23	**61.03**	18.54	4.43	23.28	**43.11**	36.10
Total n-6	**30.72**	22.27	12.35	2.08	22.06	20.87	**22.66**	10.56	4.43	**18.83**	16.51	14.48
Total n-3	2.15	4.27	21.12	**49.35**	1.69	5.36	**38.37**	7.98	-	4.44	**26.59**	21.62
n-6 : n-3	**14.30**	5.22	0.58	0.04	**13.07**	3.89	0.59	1.32	**4.43**	4.24	0.62	0.67

Table 3.3 Carcass traits of broiler chickens at day 30

	Treatments					
	LA	CA	FL	FO	SEM	p-values
Body (g)	1752.0	1650.0	1694.0	1526.0	48.02	**0.015**
Carcass (g)	1363.6	1313.5	1342.7	1216.8	36.83	**0.039**
Fat pad (g)	26.0	24.0	24.9	22.2	1.583	0.385
Breast (g)	0.29	0.26	0.26	0.25	14.79	**0.0054**
Breast:Body	0.22	0.20	0.21	0.20	0.0054	**0.048**
Breast:Carcass	0.29	0.26	0.26	0.25	0.0086	**0.042**
Adiposity (%)	1.90	1.83	1.87	1.82	0.108	0.95
Growth Rate (g/d)	9.12	10.20	10.18	10.04	0.3326	0.0836

Growth performance of young broiler chickens at day 30 with n=10 per group. Carcass (g) = Body (g) – Breast (g). Adiposity = 100 x Fat pad (g) ÷ Body (g). Growth Rate = (Day 30 Body (g) – Day 7 Body (g)) ÷ Day 7 Body (g).

CHAPTER IV
CONCLUSION

To conclude, the broiler chicken offers many advantages because it can serve as a model organism for human obesity, and diabetes studies. Further, young broiler chickens represents a research model for assessing the complex mechanisms that regulate energy homeostasis in obesity during childhood. Identifying the underlying mechanisms will provide new targets to increase in situ fatty acid oxidation and protect against childhood obesity and the harmful effects hyperlipidemia.

Cutting caloric intake is the default method employed to combat obesity, and to prevent excessive weight gain. In addition to cutting calories, we asked if white adipose tissue, devoid of brown adipocytes, could be stimulated to promote fat loss or inhibit lipid storage. A previous study from our lab reported elevated gene transcription profiles for lipid catabolism and glucose storage in adipose tissue after a short-term five hour fast, in our first study we used fasting to promote negative energy balance to assess this contention. Fasts over a short term confirmed the capability of visceral white adipose tissue to rapidly increase fatty acid oxidation locally and the positive correlation between increased fasting time and increased lipid oxidation. The implications of these findings are tremendous in that these results elucidated a mechanism in white adipose, without the oxidative capacity of brown adipocytes, to catabolize lipids locally. Additional research is needed, however, to (i) fully characterize the mechanism and its key regulators, and (ii)

to target and exploit these control points to help in reducing the incidence of obesity in humans, and fatness in chickens.

Some studies have shown that fatty acids can act as activating ligands for the PPAR nuclear receptor family for downstream regulation of energy metabolism, and others have reported that dietary n-3 PUFA can reduce fatness. Previously reported transcriptomic data from our lab showed that a short-term five hour fast caused the gene expressions of the PPAR family nuclear receptors and lipid oxidation to up-regulate in white adipose tissue. In our second study, we explored the relationship between different endogenous fatty acids, lipid storage in white adipocytes, and concomitant gene expression profiles. Using isocaloric diets enriched in different fatty acids, we confirmed that ALA, EPA, and DHA n-3 PUFAs impede fat deposition through attenuated adipocyte size in broiler chickens. Furthermore, although fat deposition and lipolysis were reduced, dietary EPA and DHA caused decreases in body and breast weights, which is not ideal for the poultry industry. Saturated dietary fatty acids altered gene expression profiles for increased adipogenesis, cell differentiation, and inhibition of lipase activity. Future research should aim towards fully characterizing the mechanism(s) that very long chain n-3 PUFAs utilize to reduce fatness.

Our first study confirmed that white adipose tissue has the capacity to oxidize fatty acids locally after a five hour fast and that this function is augmented during starvation. The second study confirmed that supplementing the diet with EPA and DHA improved

adipose tissue metabolism. This was observed through increased lipolysis, lowered body weight, and gene expression for adipocyte differentiation. Our findings do not show upregulated transcription of rate limiting genes for fatty acid oxidation, however, as suggested by increased PDK4 expression, EPA and DHA encouraged white adipose tissue to utilize fatty acids for energy.

VITA

Tania Emmanuelle Torchon was born in Port au Prince, Haiti on February 17, 1991. She and her immediate family immigrated to the United States in late 1994, and first resided in New York State. Her family then migrated to Nashville, TN in 1996 where she was enrolled to attend elementary school at Kirkpatrick Elementary. Middle and high school followed at Wright Middle and Nashville School of the Arts. Then she went on a 2 year long field trip her junior year of high school with Tennessee Governor's Academy, and graduated in May of 2009. She immediately proceeded to attend university, and in May of 2013, she received her Bachelors of Science degree from Worcester Polytechnic Institute, Worcester, MA.