12-2016

Effects of Dietary Fatty Acids on Adipose Development in Young Broiler Chicks

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To the Graduate Council:

I am submitting herewith a thesis written by Sarah Jane Howard entitled "Effects of Dietary Fatty Acids on Adipose Development in Young Broiler Chicks." I have examined the final electronic copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Animal Science.

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Effects of Dietary Fatty Acids on Adipose Development in Young Broiler Chicks

A Thesis Presented for the
Master of Science
Degree
The University of Tennessee, Knoxville

Sarah Jane Howard
December 2016

In loving memory of my grandmother, Reba "Mimi" White

Without your support and love,
I never would have found the courage to pursue this work.
I am forever grateful for your hardworking Christian example
and look forward to celebrating with you in Heaven one day.

Acknowledgments

I would like to express my sincerest gratitude to everyone who has been there for me on this incredible journey over the last two years. Without your friendship and kindness, I would not have been able to complete this degree. First and foremost, I would like to thank my parents, Eric and Angela Howard, and my brother, Austin Howard, for being there for me since day one. They have prayed for me, encouraged me, and supported me. I will never be able to thank them enough for the sacrifices that they have made in order to help me realize my dreams.

Thank you to Dr. Brynn Voy for giving me the opportunity to learn and grow under her guidance. Her enthusiasm for science is contagious and kept me going through the last two years. I am so proud to have been able to contribute to your lab. I would also like to thank Drs. Travis Mulliniks and Jun Lin for agreeing to serve on my graduate committee. Their contributions to my research and thesis were invaluable.

Words cannot express my appreciation for my teacher, advisor, colleague, and friend, Mrs. (soon to be Dr.) Emily Gray. She has been an incredible mentor throughout my undergraduate and graduate years. Thank you for serving as my home away from home and encouraging me to keep going no matter what.

Thank you to the Department of Animal Science for giving me a place to complete my research over the last two years. A special thanks to the JARTU Staff, in particular Roger and Tammy, for helping me with everything surrounding the birds and teaching me more than I ever thought I could know about avian husbandry. Robert

Dykes has been an incredible help as well with making sure that our chickens had a safe and comfortable place to stay. Thank you to all of my fellow graduate students for helping when you were needed and for providing me with the necessary social interaction to keep me sane. In particular, thank you to Yun Zhang of the Food Science department for teaching me the emulsion technique. I must also express my gratitude to Ronique Beckford for being ready to assist me with anything that I needed. I can't imagine how I could have completed this program without your willingness to help and share your expertise.

Thank you to the technicians that I had the pleasure of working with during my time in this lab. Suchita Das, thank you for being so patient and kind with me and making sure that I understood everything that we did in the lab. Jason Spence, thank you for taking the time to teach an intimidated undergraduate student how to stand on her own two feet in the lab. Thank you both for showing me how exciting and rewarding laboratory work could be.

Last but certainly not least, thank you to my undergraduate research assistants Sara Parnell, Jessie Tipton, and Hannah Jernigan. These ladies were so helpful with caring for our chickens and were eager to learn new lab techniques whenever possible. I hope that this experience has inspired you to consider taking on more opportunities in research.

Abstract

The broiler chicken is an attractive model for human obesity, and childhood obesity in particular, due to its ability to eat independently at hatch, put on abdominal fat post-hatch, and its similarities in lipid metabolism. Three studies are presented to investigate the potential for omega-3 fatty acids administered *in ovo* and in the diet at hatch to alter adiposity. Studies one and two investigated manipulating the fat source in the diet from hatch to days 14 and 24, respectively. Oils tested included corn, lard, macadamia, tuna, fish, safflower, flaxseed, and coconut. Data concerning body weight, breast weight, and abdominal fat weight were measured. In addition, basal lipolysis, PPARɣ expression, and ex vivo lipolysis and adipocyte differentiation were explored. The third study was the first known experiment to attempt *in ovo* injections of lipids at day 17.5 of incubation, and focused on improving the technique for fatty acids. The oils tested for *in ovo* injections were corn oil and fish oil, though the experiment was cut short due to neurological issues. The first two studies confirm that enriching the diet in omega-3 polyunsaturated fatty acids can be used to decrease adipocyte size and differentiation, which could potentially provide benefits to both broiler chickens and humans.

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Chapter 1. Review of Literature

1.1 Human Obesity

When adipose tissue is accumulated and stored in excessive amounts, the subject is referred to as "obese." In humans, this is defined as a body mass index of greater than or equal to 30.0 kg/m^2 (CDC, 2015). Obesity has become an epidemic in the United States, with approximately 35% of adults in the United States being reported as obese in 2012 (Ogden et al., 2014). However, this number is likely much greater as only about 9,000 participants were surveyed and it was based on self-reported data. This number also does not include participants that are merely considered "overweight," which has its own consequences related to excess adiposity. There are many health risks associated with obesity including, but not limited to, hormone imbalances, cancer, type 2 diabetes, cardiovascular disease, shortness of breath, sleep apnea, and osteoarthritis (Visscher & Seidell, 2001). Health complications due to obesity cost $1,429 more per person per year than the cost of healthcare for a person of healthy weight (Finkelstein et al., 2009).

Three main factors can contribute to human obesity: diet, exercise, and genetics. Diet and exercise are the two traits that can quickly be manipulated. As long as calories consumed is less than or equal to calories used, weight gain generally will not occur. Calories consumed can be reduced by monitoring what is eaten, while calories burned can be increased by adding extra exercise into daily routines (CDC, 2015).

1.2 Childhood Obesity

Although obesity can have many implications in adult humans, it can cause problems for children as well. Obesity is measured slightly differently for children. The same Body Mass Index that is used for adults is measured, and it is plotted against age on a BMI-for-age percentile graph (OAC, 2016). There are separate graphs for boys and girls, so sex is factored in as well. In children, complications such as hyperlipidemia, glucose intolerance, and early maturation (Dietz, 1998). It can also cause psychosocial issues that cause children to become preoccupied with their bodyweight and physical appearance (Dietz, 1998). Children that are obese are much more likely to become obese adults, which could lead them down a very destructive path in regards to their health (Whitaker et al., 1997).

Similarly to what is known about adults, diet, exercise, and genetics are contributors to childhood obesity. Growing technology and an increase of electronic devices in the home is also a contributing factor. When children are watching television, they have less time to potentially spend exercising, which limits the physical activity that they are able to perform each day. Children are more likely to request the foods seen in television commercials, and typically these commercials consist of highly processed and unhealthy foods, not things like lean protein, fruits, and vegetables (Taras et al., 1989). Consuming foods that are often seen in television commercials that have high energy density, low fiber, and high fat content increases the likelihood for the consumer to become obese (Johnson et al., 2008). One study reported that children who watched

television for one hour had a significantly lower resting metabolic rate than those who stared at a blank television set for the same amount of time (Klesges et al., 1993).

In addition, environmental factors can have an impact on excess fatness. Children who are raised in lower-income homes are more likely to become obese (CDC, 2015). In addition, parental education level has an effect on obesity, as children of parents who did not complete high school are much more likely to become obese than children of parents who completed a college education (9% vs 19% among girls; 11% vs 21% among boys in 2010) (CDC, 2015). Parents can also influence a child's health. When children eat in restaurants, they are more likely to consume excess calories than what they would normally consume at home (Zoumas-Morse et al., 2001). Parents set an example for what their children should eat, so family meals together can be very influential. Families that eat together are more likely to consume fruits and vegetables and less fat (Gillman et al., 2000). Mothers and daughters show a moderately strong correlation between their diets, indicating that mothers could be significantly influencing what their children choose to eat (Oliveria et al., 1992).

All of these things considered, it is very difficult to perform obesity research on children, particularly when it comes to manipulating the diet. Often, parents are not willing to place their children on experimental diets for fear that it may harm their development. Additionally, parents must sign waivers and consent to the research, as children under 18 years old have not reached the legal age on consent for themselves (HHS, 2016). It is still questionable as to whether parents should ethically be allowed to

make the decision to participate in research for their children, which is why children who can understand the basics of the research must be willing to consent as well. This does not cover children who are too young to comprehend the study for which they are being considered.

1.3 Excess Adiposity in Broiler Chickens

Excess fatness is not only a problem in humans or even just mammals. It has also been shown to also have a negative impact on the health of avian species. Chicken has quickly become the most commonly consumed source of animal protein in the United States, with the average person eating approximately 38.5 kg of chicken in 2014 ("National Chicken Council," 2016). Since 1992, chicken has been consumed at a greater rate than that of pork and beef, which were consumed at a rate of 24.5 kg and 22.7 kg per person for the year 2014, respectively ("National Chicken Council," 2016). As the demand for chicken has increased, producers have been forced to find ways to increase production, which could be accomplished through producing more chickens or producing chickens that reach the market weight faster.

The Chicken-of-Tomorrow program was started in 1945 in order to develop the broiler industry (Shrader, 1952). The goal was to make a more efficient bird for producing meat. The birds were improved through selective breeding and improved feed quality. Chickens in 1945 reached market age at approximately 84 days and weighed in at an average of 1.4 kg., while modern broilers go to market at 48 days and weigh an average of 2.8 kg. ("National Chicken Council," 2016). Additionally, in 1945,

chickens consumed 1.8 kg of feed in order to gain 0.5 kg of body weight, whereas modern broilers only need to consume 0.9 kg of feed to gain 0.5 kg ("National Chicken Council," 2016).

With the increase in bodyweight, however, the amount of the fat in the bird increased as well, particularly in the abdominal region (Havenstein et al., 2003). This is particularly intriguing due to the fact that an average broiler chicken diet consists of only about two percent fat, which is very low compared to most species (ACMF, 2013). One study found that birds from a 1957 strain had less than half the abdominal fat as a percentage of bodyweight than birds from a 2001 strain (Havenstein et al., 2003). When feed is converted to fat instead of muscle, money is essentially wasted. Consumers have indicated that appearance is the main determining factor when purchasing meat at grocery stores (Kennedy et al., 2004). One quality of appearance that they find important is the amount of fat on the piece of meat (Kennedy et al., 2004). Fat is mostly trimmed off during production, which takes more time for the workers when the chickens are being processed.

There are several known causes of excess fatness in broiler chickens. Most fat is synthesized through the process of de novo lipogenesis in the liver (Griffin et al., 1992). From the liver, fatty acids are redirected to the adipocytes by very low density lipoprotein. Then, lipoprotein lipase controls uptake into the cells. Previous studies have used very low density lipoprotein levels to select for high or low fatness in divergent chicken lines (Wang et al., 2007). On the other hand, chickens that have been selected

for high or low fatness have been found to have high or low very low density lipoprotein levels as well (Ji et al., 2012). This indicates that manipulating very low density lipoprotein levels could be a potential method for limiting obesity, as presence of this lipoprotein is reflective of fatty acid mobilization.

Broilers are an under-used but potentially valuable model organism for studies of human obesity. Chickens rely on the liver for the majority of their lipid synthesis, like humans (Leveille et al., 1975), but unlike more widely-used rodent models. Broiler chickens deposit excess fat primarily in the abdominal depot, which makes up about 1.5% of the final carcass weight (Havenstein et al., 2003). This depot anatomically resembles visceral adipose tissue in humans. Visceral fat is of particular concern for human obesity because it is the depot that is primarily associated with metabolic dysfunction and increased risk for Type 2 diabetes and cardiovascular disease. Broiler chicks are an especially attractive model for studies of childhood obesity. The diet of the chicken can be manipulated immediately upon hatching, unlike rodent models which must be weaned before their diet can be directly altered. In broiler chickens, subcutaneous fat is present at approximately 12-14 days of incubation (Liebelt & Eastlick, 1954). Conversely, abdominal fat is almost nonexistent at hatch. It begins to exponentially develop beginning around day five after hatching (Tzeng & Becker, 1981). Therefore, there is the potential to modify this critical fat pad as it develops.

1.4 Adipose Tissue Biology

Adipose tissue is comprised of adipocytes, preadipocytes, fibroblasts, endothelial cells, and multipotent stem cells (Moreno-Navarrete & Fernández-Real, 2012). Mature adipocytes make up about 30% of this tissue. Adipocytes, commonly known as fat cells, can be found throughout the body between the skin and muscle layers. They contain a single lipid droplet, which makes up most of the cell, and a nucleus and cytoplasm.

Most of the fatty acids from the diet come in the form of triglycerides, which are not absorbed by the human intestine. The fats are emulsified in the small intestine by bile salts and pancreatic lipase breaks them down into mono-glycerides and di-glycerides. The combination of bile salts and the products of triglyceride breakdown form mixed micelles. In the enterocytes, micelles are resynthesized into triglycerides and packaged into chylomicrons, where they are released into blood circulation. Lipoprotein lipase on the surface of adipose tissue breaks down the chylomicrons into free fatty acids and glycerol. The fatty acids are then absorbed by the adipocytes and the glycerol is removed from the circulation by the liver. Once inside the adipocytes, fatty acids are re-esterified into triglycerides.

Growth of adipose tissue can be caused by either hyperplasia, hypertrophy, or a combination of the two. Hyperplasia is an increase in adipocyte number, while hypertrophy is an increase in adipocyte size. Hyperplasia is more strongly correlated with severity of obesity (Hirsch & Batchelor, 1976). Hyperplasia occurs when mesenchymal stem cells develop into preadipocytes and then into mature adipocytes.

Many mechanisms contribute to adipocyte differentiation, but peroxisome proliferator-activated receptor gamma (PPARɣ) is the major regulator of adipogenesis (Henry et al., 2012). PPARɣ is a transcription factor which controls the peroxisomal beta-oxidation pathway of fatty acids and increases differentiation. On the other hand, hypertrophy typically precedes hyperplasia, and hypertrophy is a risk factor for obesity-related diseases such as type 2 diabetes (Weyer et al., 2000). Hypertrophy occurs in times of energy surplus, where fatty acids accumulate and are re-esterified into triacylglycerol, which is taken up by adipocytes to increase lipid droplet volume (Frühbeck et al., 2014).

1.5 Fatty Acids

Lipids are the most energy-dense macronutrient that can be consumed, with nine kilocalories of energy being provided for each gram of lipid, whereas carbohydrates and proteins both possess 4 kilocalories of energy per gram. Current dogma suggests that diets high in lipids are responsible for the obesity epidemic, however it is now thought that the type of fat is a better predictor of obesity than fat in general. For example, increased intake of saturated fats in particular has been associated with increased likelihood of developing obesity and obesity-related diseases (Dayton et al., 1966). Conversely, isocaloric diets that are enriched in polyunsaturated fats lead to a lower basal metabolic rate, and thus a lower propensity for weight gain (Jones & Schoeller, 1988).

Most dietary lipids are made up of fatty acids. A fatty acid is a molecule made up of a carboxylic acid head and an aliphatic acyl chain tail (Stipanuk & Caudill, 2013). Fatty

acids can be classified as saturated or unsaturated based on the location and number of double bonds present in their chemical structure. Saturated fatty acids have no double bonds, while unsaturated fatty acids have at least one double bond. Unsaturated fatty acids with only one double bond are called monounsaturated. Fatty acids with more than one double bond are called polyunsaturated. Classification of polyunsaturated fatty acids, or PUFA, can be further broken down into omega-3 and omega-6 fatty acids, which are named based on the location of their first double bond from the methyl end of the fatty acid chain. Saturated fatty acids and omega-6 polyunsaturated fatty acids are often found in animal products and vegetable oils, whereas monounsaturated fatty acids and omega-3 fatty acids are commonly found in fish, nut, and seed oils (Tvrzicka et al., 2011). Although they can both be classified as polyunsaturated fatty acids, omega-3 fatty acids and omega-6 fatty acids have very different physiological funtions. Omega-6 fatty acids produce excessive amounts of the pro-inflammatory eicosanoids prostaglandin E_2 and leukotriene B_4 (James et al., 2000). Omega-3 fatty acids like eicosapentaenoic acid and docosahexaenoic acid have the opposite effect, and can have more of an anti-inflammatory effect (Simopoulos, 1999).

Omega-3 fatty acids have been shown to have positive effects on human health in regards to obesity. One study showed that increasing levels of omega-3 intake could increase satiety in obese individuals that were attempting to lose weight through calorie restriction when compared to calorie restriction alone (Parra et al., 2008). Another experiment showed that fish oil combined with exercise decreased fatness and

increased lean tissue in humans when compared to either fish oil or exercise alone (Hill et al., 2007). Consumption of at least 35 g of fish had a relative risk of death from coronary heart disease of 0.62 and a relative risk of nonsudden death from myocardial infarction of 0.33, both of which are possible side effects of obesity (Daviglus et al., 1997).

The benefits of omega-3 fatty acids have also been shown in rodent models. A study in mice indicated that omega-3 supplementation in high fat diets can prevent the development of obesity when compared to high fat diets alone (Ruzickova et al., 2004). This was also observed when rats were fed high fat diets with varying fatty acid compositions. The rats fed a combination of lard and fish oil displayed 20% less subcutaneous fat and 30% less visceral fat compared to rats fed a lard diet and a lard and corn oil combination diet (Hainault et al., 1993). When diets high in omega-6 fatty acids were substituted with just 6% omega-3 fatty acids, insulin resistance, a common side effect of obesity, was prevented in rodents (Storlien et al., 1987).

1.6 In Ovo Injections

Embryonic development and the first several days after hatch are extremely important to the chick's outcome, as this time period often comprises more than one third of the animal's lifespan when it goes to slaughter. During the last three to four days of embryonic development, the digestive tract of the growing chick increases in size exponentially (Z Uni et al., 2003). The chick then begins to consume its first meal, the surrounding albumin. Therefore, the first opportunity to manipulate the chick's diet

is when it consumes this albumin while it is still growing within the egg. This method is known as *in ovo* supplementation, and involves insertion of a needle into the egg in order to deliver a dose of medication or nutrient to the bird.

In ovo injections have previously been used to improve various aspects of the chick at and after hatching. One group did a variety of *in ovo* injections on day 17.5 of embryonic development and concluded that *in ovo* supplementation could potentially increase hatchability and decrease mortality after hatch (Z. Uni & Ferket, 2004). They proposed that *in ovo* injections consisting of either a carbohydrate solution (maltose, sucrose, dextrin, and NaCl), β-hydroxy-β-methylbutyrate, or a combination of the two could be used to increase intestinal villi length, which allows for better absorption of nutrients by the chicken (Tako et al., 2004). The carbohydrates were used due to previous research which concluded that decreasing the time between hatching and access to feed can accelerate intestinal development (Noy & Sklan, 1998). β-hydroxy-β-methylbutyrate was chosen based on research that determined that this it decreases chicken mortality, increases carcass yield, and plays an important role in protein metabolism in muscle, mainly in attenuating proteolysis (Nissen et al., 1996). Later, injections of maltose, sucrose, dextrin, and HMB were used to increase the birds' weight (and in particular, breast weight) at hatch, which can lead to larger birds as adults, and in turn can allow for a larger meat yield and higher profits for producers (Z. Uni et al., 2005).

In addition, *in ovo* injections have previously been used to alter adiposity. Injections of at 0.2 mg of anti-adipocyte monoclonal antibodies on day 15 of embryonic development led to decreased abdominal fat at 42 days of age (Wu et al., 2000). One study investigated *in ovo* injections of selenium in 8 day old embryos and determined that this increased adipose tissue mass by 30% (Hassan et al., 2014). Ovine growth hormone *in ovo* injections administered on day 11 of embryonic development were determined to increase adipocyte size and decreased sensitivity to glucagon induced lipolysis when it was measured in the 7 week old broilers (Hargis et al., 1989). There are no studies at this time that have investigated the effect of *in ovo* injections of fatty acids on adiposity in broiler chickens.

Chapter 2. Omega-3 Fatty Acids Decrease Adipocyte Size in Young Broiler Chicks

2.1 Abstract

Obesity is a growing problem in humans as it causes many detrimental health problems. Additionally, excess fatness has become an issue in broiler chickens, or chickens raised for meat, as fat does not return the same profits as muscle for producers. Broiler chickens are a novel yet promising model for human obesity with similarities in fatty acid metabolism and their ability to readily deposit abdominal adipose tissue post-hatch. Thus, day old broilers were used to investigate the hypothesis that enriching the diet at hatch in long chain polyunsaturated fatty acids at hatch could reduce adiposity. Chicks were fed isocaloric diets which contained 3% fat sources of corn oil, flaxseed oil, macadamia nut oil, safflower oil, fish oil, or tuna oil. Weight gain ($P = 0.392$), relative breast weight ($P = 0.22$), relative abdominal fat pad weight ($P = 0.708$), lipolysis ($P = .083$), and serum glucose levels ($P = 0.055$) were maintained across diets. Average adipocyte size was greatest in chicks fed the corn oil diet and least in the chicks fed the tuna oil diet ($P < 0.0001$), indicating that adiposity could potentially be altered at the cellular level.

2.2 Introduction

In the United States, obesity has become an increasingly problematic issue for human health. As of 2012, approximately 35% of adults and 17% of children were reported as being obese (Ogden et al., 2014). Obesity causes increased risk of many health problems, including cardiovascular disease (2-3 fold increase), sleep apnea (4 fold increase), type 2 diabetes (10 fold increase), and certain types of cancers (1.5-3 fold

increase in endometrial cancer, 1.8 fold increase in breast cancer) (Visscher & Seidell, 2001). Diet is one of the main factors that contributes to obesity. When calories consumed is greater than calories used by the body, weight gain can and will occur. In addition to calories, the diet source can have an impact on weight gain. Consuming a diet rich in energy-dense foods that are low in fiber and high in fat can increase the likelihood of obesity (Johnson et al., 2008). Overweight or obese preschoolers are five times more likely to develop into obese adults, therefore it is critical that excess fatness be prevented in children in order to avoid obesity in adulthood ("Obesity Society," 2014)

Excess fatness is also a problem in broiler chickens, or chickens raised for human consumption of their meat. In an effort to meet increases in demand, chickens have been bred to reach market weight more rapidly. Consequently, the deposition of fat increased as well (Havenstein et al., 2003). When feed is converted to fat instead of muscle, potential profits are lost. Fat is trimmed off during production, which leads to increased labor costs and a reduction of profit since most of the fat is thrown away or sold at a much lower price than meat.

Broiler chickens are novel yet promising models for human obesity. They possess similarities in some aspects of lipid metabolism, such as the liver as the main site of lipid synthesis and depositing a high percentage of excess fat in the abdominal region (Leveille et al., 1975). The ratio of omega-3 fatty acids to omega-6 fatty acids has been a recent focus in regards to the obesity epidemic, particularly in humans. Studies in mouse models have shown that diets enriched in omega-3 fatty acids can prevent the

development of obesity (Ruzickova et al., 2004). One study compared omega-3 fatty acid diets to tallow-based diets in broiler chickens, and reported that the omega-3 fatty acid diet caused a decrease in adipocyte size (González-Ortiz et al., 2013). Thus, we want to determine the differences in adipose tissue development between chicks fed diets with varying levels and types of omega-3 and omega-6 fatty acid based diets.

2.3 Materials and Methods

Subject Selection and Assignment of Treatments

One hundred eighty fertilized broiler-type chicken eggs were obtained from the Pilgrim's poultry plant in Chattanooga, Tennessee. Sixty eggs were placed in an incubator at 37.5˚C and 50% relative humidity each day for three consecutive days. A timeline of three days was used in order to give ample time for the most precise dissections possible at the conclusion of the study. Upon hatching, chicks were weighed and randomly assigned to one of six pens, each of which was assigned a different diet: safflower oil, tuna oil, fish oil, macadamia oil, flaxseed oil, or corn oil. The diets were named based on the fat source.

Preparation of Diets

All diets were made using a standard starter diet recipe, with only the fat source being altered. Chicks had ad libitum access to feed and water for the entirety of the experiment. Diet formulation is reported in Table 2.

Termination of Study

At 14 days of age, the chicks were euthanized using carbon dioxide asphyxiation. At euthanasia, the final body weight was measured. Blood was collected postmortem via cardiac puncture with an 18 ga needle and 3 mL syringe. Subcutaneous fat was removed from the thigh region. The entire abdominal fat pad and breast muscle were removed and weighed. Samples of subcutaneous fat, abdominal fat, liver, and breast muscle were snap frozen in liquid nitrogen and stored at -80˚C until analysis.

Blood Serum Analysis

Blood collected at euthanasia was transferred into 2mL Eppendorf tubes that were centrifuged at 2,000 x g for 10 minutes and serum was then collected from the top layer. Glucose was measured using Thermo Fisher Scientific's Infinity Glucose (catalog number TR15421, Waltham, MA). Non-esterified free fatty acid concentrations were measured for 12 birds from each diet using Wako Diagnostics' HR Series NEFA-HR(2) kit (catalog numbers 999-34691, 995-34791, 991-34891, 993-35191, and 276-76491, from Richmond, VA).

Measurement of Adipocyte Size

Two chickens per diet were selected for the measurement of adipocyte size. Abdominal white adipose tissue was removed and collagenase digestion was performed using type 1 collagenase in PBS with 10 mM glucose. The minced tissue was rocked in the collagenase for 1.5 hours at 37˚C. Aliquots of the floating adipocyte layer were removed in order to take pictures. Approximately 20 um of the adipocyte layer was

pipetted onto a microscope slide and a glass slip was applied. Photos were taken using 20x magnification with an Advanced Microscopy Group EVOS XL Core microscope (Fisher Scientific, Pittsburgh, PA). The images were analyzed using ImageJ 1.49V software (National Institute of Health) to determine the average adipocyte size for each diet.

Statistical Analysis

Statistical analyses were performed using SAS 9.4 software (Cary, NC). The design used was randomized complete block design with diet as the fixed effect and day as the random effect. Results were considered statistically significant with a P value of less than 0.05. Significance levels and standard deviations for each set of data are represented within their respective figures.

2.4 Results

There was no significant difference between diets for body weight gain (Figure 2.1, P = 0.392). There was also no significant difference in percentage breast weight of total body weight (Figure 2.2, P = 0.22) or percentage abdominal fat pad weight of bodyweight (Figure 2.3, P = 0.708). There was also no significant difference based on non-esterified fatty acid concentration (Figure 2.4, P = 0.083).

There was no significant difference in glucose concentration (P = 0.055), but trends were observed. Corn and safflower oils had greater glucose concentrations when compared to tuna, fish, and macadamia oils (Figure 2.5). Chicks that received the tuna

oil diet had lower glucose concentrations than chicks fed corn oil, flaxseed oil, and safflower oil.

As corn oil served as the control fat source for this study, data for adipocyte size is represented by fold change compared to corn oil in Figure 3.6. The chicks on the tuna oil based diet showed the greatest decrease in size when compared to corn oil, with a fold change of 0.24. Fish was the most similar to corn with a fold change value of 0.88.

2.5 Discussion

Although omega-3 dietary fatty acids did not have a profound effect on elements of broiler chicken growth such as weight gain, breast weight, and abdominal fat weight, there were some interesting findings at the cellular level. Glucose measurements were not statistically significantly different, but the trends indicated that chickens fed diets rich in omega-3 fatty acids had lower glucose concentrations than the other diets. This could be caused by an increase in insulin sensitivity due to the greater level of omega-3 fatty acids in the diet (Popp-Snijders et al., 1987).

Preliminary studies from our lab have shown that omega-3 polyunsaturated fatty acids can be used to reduce fatness in adult (30 day old) broiler chickens, but the present study, which used young broiler chicks, did not achieve the same results (Torchon, 2015). However, the average adipocyte size was smaller in chicks fed the macadamia and tuna oils. This could indicate that at the young age of two weeks, chicks have not had time to develop enough abdominal fat to show a vast difference in fatness.

Perhaps extending this study by a few weeks would show more profound differences in abdominal fat like those seen in broiler chickens fed these diets in adulthood.

2.6 Appendix: Tables and Figures

Table 2. Diet formulation

Feed Description	As-fed lbs. per 100 lb. bag
Corn meal	61.7
Soybean meal	32.2
DL Methionine	0.25
Salt	0.35
Limestone	1.10
Dicalcium phosphate	2.10
Vitamin Pre-Mix	0.25
Fat	1.90
Coban	0.05
Lysine	0.10

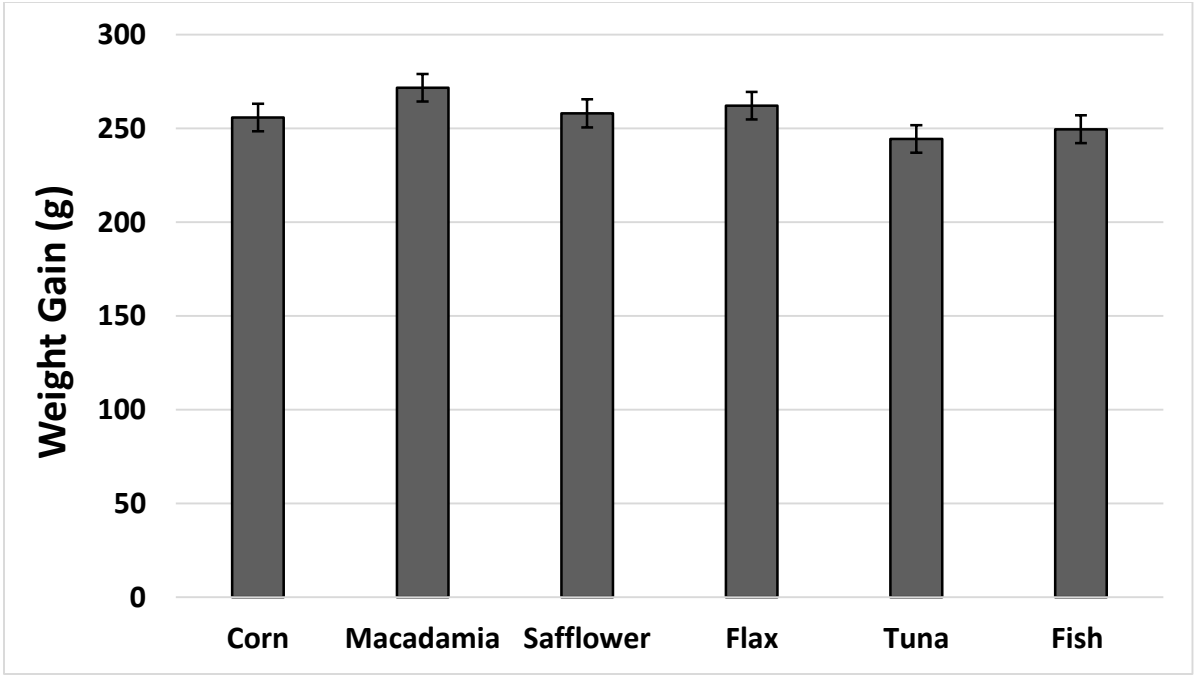

Figure 2.1. Comparison of weight gain across diets. Weight gain was calculated as final body weight at euthanasia minus the body weight at hatch. Analysis of variance was performed (n = 143, P = 0.392).

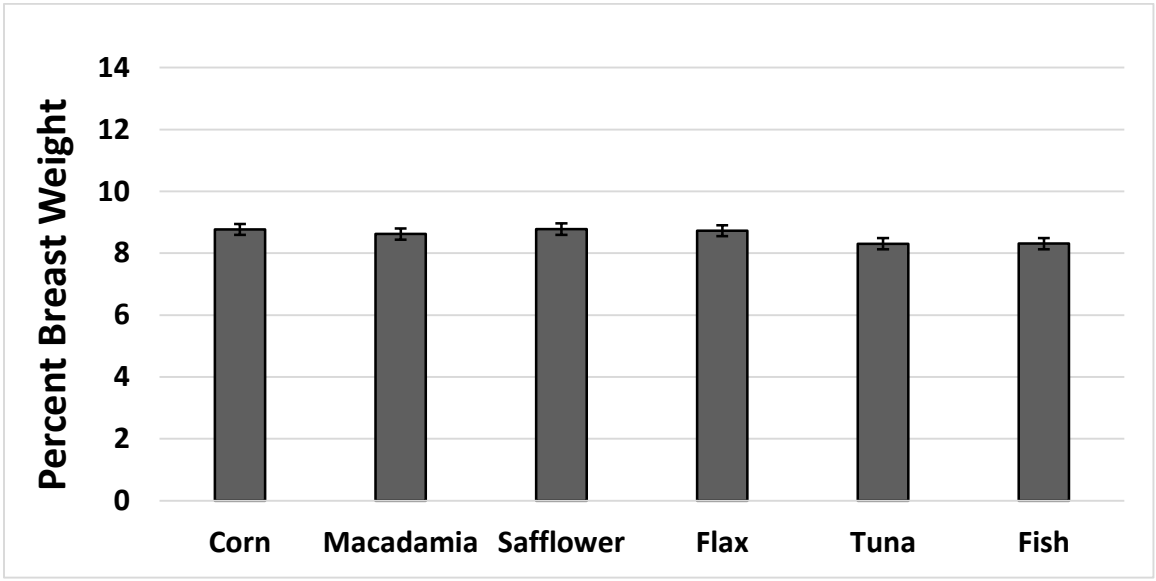

Figure 2.2. Comparison of breast weight. This was calculated as breast weight divided by bodyweight at euthanasia, converted to percentages. Analysis of variance was performed (n = 143, P = 0.22).

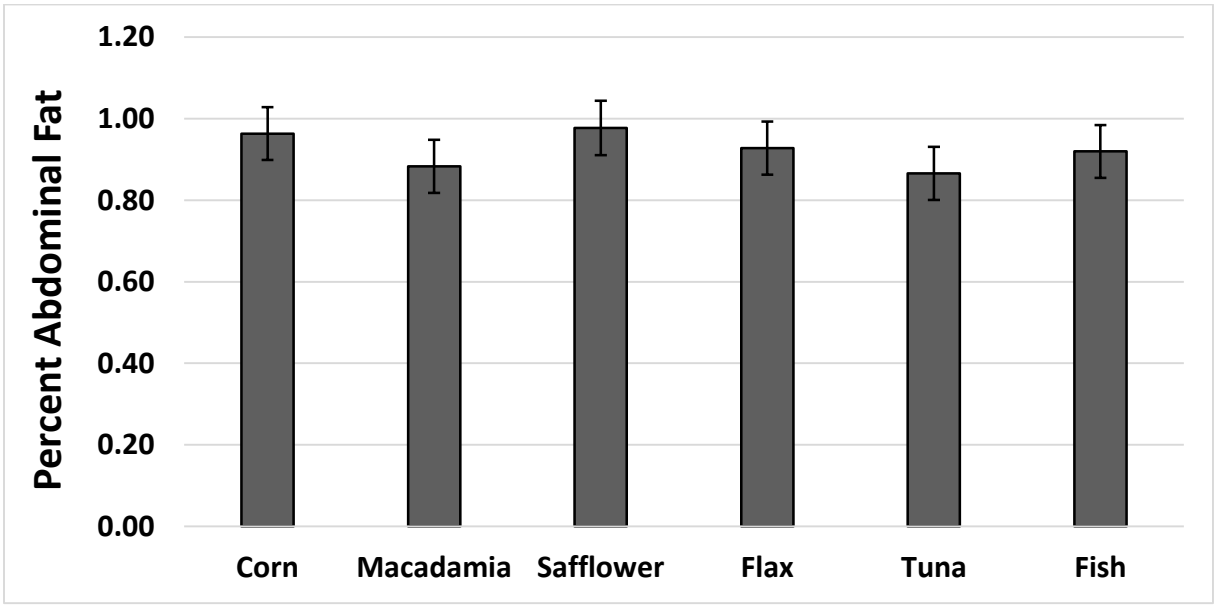

Figure 2.3. Comparison of abdominal fat pad weight as a percentage of bodyweight at euthanasia (n = 143, P = 0.708).

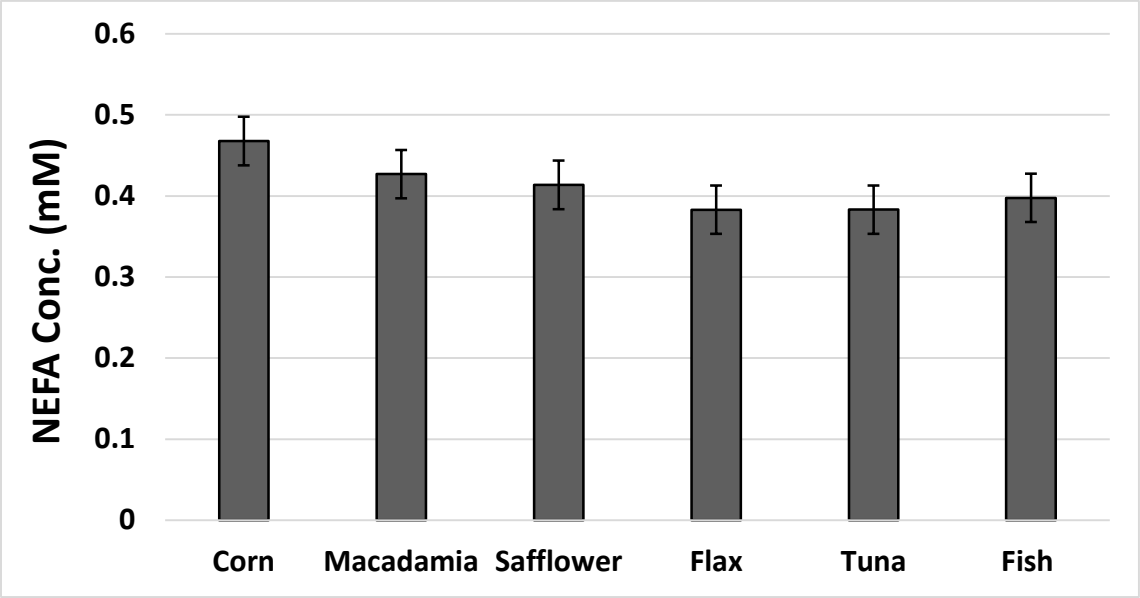

Figure 2.4. Comparison of non-esterified fatty acid concentrations. Analysis of variance was performed (n = 72, P = 0.083).

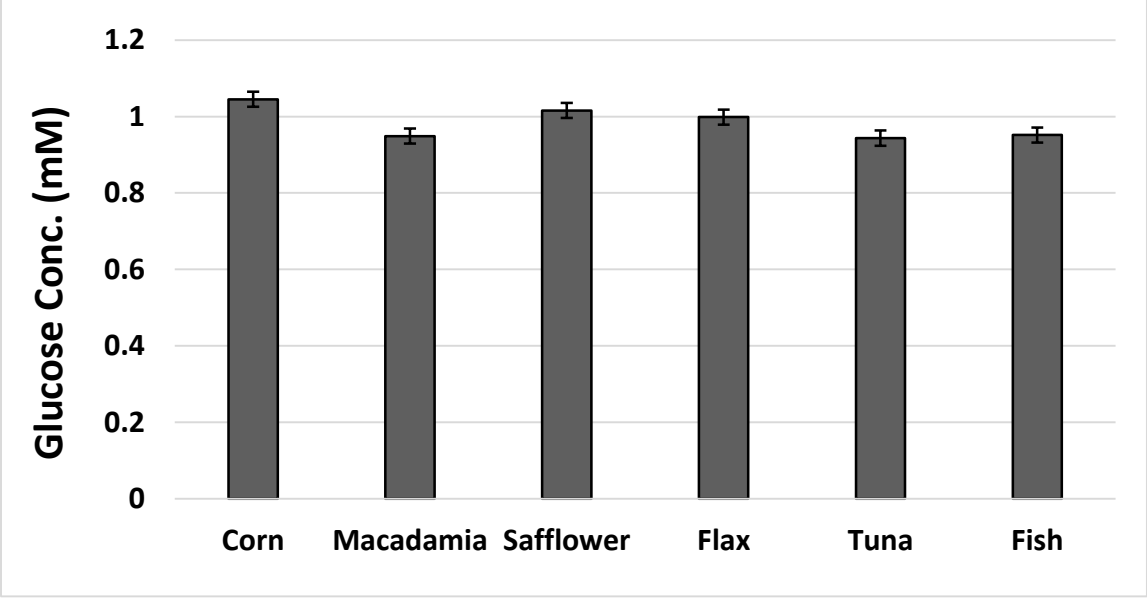

Figure 2.5. Comparison of glucose concentrations. Analysis of variance was performed (n = 64, P = 0.055).

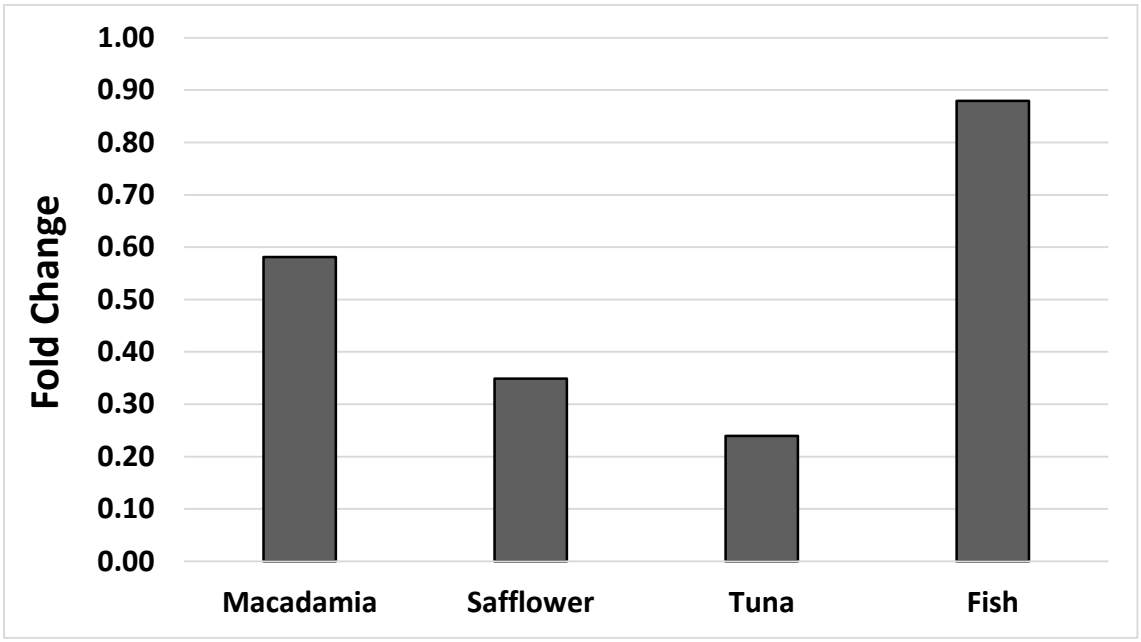

Figure 2.6. Comparison of adipocyte size across diets using fold change compared to the control diet of corn oil ($P < 0.0001$).

Chapter 3. Effects of Omega-3 Fatty Acids on Adipose Development in Young Broiler Chicks

A version of this chapter will be submitted for publication by Sarah Howard and Brynn Voy to the Journal of Nutritional Biology. This article will be revised by Brynn Voy and the final draft will be submitted for publication. The primary authors will be Sarah Howard, Brynn Voy, and Suchita Das.

Associated figures and tables are included in an appendix at the end of the chapter.

3.1 Abstract

Obesity causes numerous health problems for both adults and children. Additionally, excess fatness in meat chickens, also known as broilers, causes profit loss as producers are unable to sell fat for the same price as muscle. Broiler chickens, which readily deposit abdominal adipose tissue post-hatch and share similarities in fatty acid metabolism with humans, were used to investigate the hypothesis that enriching the diet at hatch in long chain polyunsaturated fatty acids at hatch could reduce adiposity. Chicks were fed isocaloric diets which contained 3% fat sources of lard, coconut oil, macadamia nut oil, fish oil, or tuna oil. While weight gain, relative breast weight, and relative abdominal fat pad weight were maintained across diets, average adipocyte size was decreased ($P < 0.0001$) by feeding the tuna and fish oil diets, which are enriched in omega-3 fatty acids. There was no difference in lipolysis levels, however, adipocyte differentiation was significantly decreased in the fish and tuna oil diets ($P < 0.0001$). PPARɣ was investigated as a potential mechanism for the difference in adipocyte differentiation, but no significant difference was found in this gene expression. This

study indicates that diet has the potential to lessen the effects of obesity at the cellular level.

3.2 Introduction

In the United States, nearly 70% of adults and 30% of children were labeled as overweight or obese (body mass index greater than 25) as of 2012 (CDC, 2015). Excess fat causes many health problems including Type 2 diabetes, cardiovascular disease, hypertension, and cancer (Visscher & Seidell, 2001). However, humans are not the only species affected by the obesity epidemic. Broiler chickens, or chickens raised for meat consumption, are also prone to develop excess fat. When chickens prioritize their nutrients into producing fat instead of lean muscle, the nutrients are essentially wasted due to the removal of fat during processing. In addition, chickens are an ideal model for human obesity because of their shared primary location of lipid synthesis (liver) and their predilection to accumulate excess fat primarily in the abdominal region (Leveille et al., 1975). Chickens also have the advantage over rodent models in the fact that they can eat independently at hatch and do not rely on nutrition from the mother. In order to manipulate the diet of infant mammalian models, the mother must consume the nutrients, the nutrients must make it into the milk supply, and the offspring must consume the milk, which is a more difficult process to monitor than feeding the infant itself.

Dietary lipids have been shown to impact the likelihood of developing obesity in many species. In particular, omega-3 fatty acids have become of particular interest for

researchers as they have decreased some obesity-related issues in humans. For example, chickens that were fed a diet enriched in omega-3 fatty acids had a decreased fat pad weight than those fed a diet with more saturated fats (Newman et al., 2002). In humans, women who took a fish oil supplement had greater weight loss than those who relied on diet and exercise alone (Munro & Garg, 2013). At this time, no studies have been conducted to look at the effect of omega-3 fatty acids in newly hatched to juvenile broiler chicks. Because broiler chickens have such a brief time before they go to market, it is critical that excess fatness be prevented from the beginning of their lives.

3.3 Materials and Methods

Subject Selection and Assignment of Treatments

One hundred one day old broiler-type chicks of mixed sex were obtained from the Hubbard hatchery in Pikeville, Tennessee. Chicks were weighed individually and randomly assigned to one of five treatment groups: macadamia nut oil, tuna oil, fish oil, coconut oil, or lard. Treatment groups were named based on the primary source of fat in the diet.

Preparation of Diets

All diets were made using a standard starter diet recipe, with only the fat source (3% of the diet) being altered. Chicks were fed ad libitum and had unlimited access to water for the entirety of the experiment. Diet formulation is seen in Table 3.

Termination of Study

At 24 days of age, the chicks were euthanized using carbon dioxide asphyxiation. At euthanasia, the final body weight was measured. Blood was collected postmortem via cardiac puncture with an 18 ga needle and 3 mL syringe. Subcutaneous fat was removed from the thigh region. The entire abdominal fat pad and breast muscle were removed and weighed. Samples of the abdominal fat (approximately 2 g) were preserved in 4% paraformaldehyde in 0.1 molar sodium phosphate buffer at pH 7.4 for 24 hours and were then placed in 70% ethanol for later adipocyte size measurements. Samples of subcutaneous fat, abdominal fat, liver, and breast muscle were snap frozen in liquid nitrogen and stored at -80˚C until analysis.

Blood Serum Analysis

Blood collected at euthanasia was transferred into tubes that were centrifuged at 2,000 x g for 10 minutes and serum was then collected from the top layer and placed in an Eppendorf tube. The serum samples were stored in a -80˚C freezer until analysis. Glucose was measured using Thermo Fisher Scientific's Infinity Glucose (catalog number TR15421, Waltham, MA). Non-esterified free fatty acid concentrations were measured using Wako Diagnostics' HR Series NEFA-HR(2) kit (catalog numbers 999-34691, 995-34791, 991-34891, 993-35191, and 276-76491, from Richmond, VA).

Measurement of Adipocyte Size

Two birds were randomly selected from each diet group for measurement of adipocyte size. Samples of abdominal fat that had been preserved in 4%

paraformaldehyde in 0.1 molar sodium phosphate buffer at pH 7.4 and then 70% ethanol were sent to Jim Wesley at Ridge Microtome (Knoxville, TN), who dehydrated the samples, removed the alcohol with a hydrophobic clearing agent, and infiltrated the adipose tissue with molten wax. Tissues were then sliced in a microtome and mounted to a glass slide, then treated with H&E staining. Two slides were produced per bird, and five pictures were taken of each slide at 20x magnification using an Advanced Microscopy Group EVOS XL Core microscope (Fisher Scientific, Pittsburgh, PA). The images were analyzed using ImageJ 1.49V software (National Institute of Health) to determine the average adipocyte size for each diet.

PPARɣ Expression

Five birds were randomly selected from each diet to measure PPARɣ expression. Ribonucleic acid (RNA) was extracted using a Qiagen RNeasy Lipid Tissue Mini Kit and RNA was quantified using a spectrophotometer (Qiagen, Hilden Germany). The quality was also checked using gel electrophoresis and then cDNA was made using an iScript kit (Bio-Rad, Hercules, CA). The cDNA was used to perform RT-PCR using 18S to ensure the quality of the cDNA, and it was analyzed for purity using gel electrophoresis. Finally, QPCR was performed to measure the relative gene expression of PPARɣ and was compared with a housekeeping gene, TBC. ΔCq was calculated by subtracting TBC expression from the PPARɣ expression. Then, an arbitrary expression value was calculated for each diet by raising 2 to the negative ΔCq.

Adipocyte Differentiation

Samples of abdominal fat from chicks from the lard, tuna, macadamia, and fish oil diets weighing approximately 1 g were digested in 10 mg collagenase and 3 µmoles of glucose per mL with shaking at 37˚C. This was done to isolate the stromal vascular fraction, or the portion of adipose tissue rich in preadipocytes (Rodbell, 1964). The suspension was centrifuged for 1 minute at 400 x g. The stromal vascular fraction, which was sedimented at the bottom of the centrifuge tube, was placed into two 24-well plates after cells were counted to ensure that approximately 0.18 x 10^{-6} preadipocytes were placed in each well. One plate was allowed to spontaneously differentiate. The other plate underwent induced differentiation by treating the media (Roswell Park Memorial Institute medium, chicken serum, and Fungizone) with oleic and linoleic acid (Sigma-Aldrich, St. Louis, MO, catalog #L96655). After 48 hours, the plates were stained with 250 µL Oil Red O per well and were quantified with a spectrophotometer at a wavelength of 490 nanometers.

Glucagon Challenge Study

Samples of abdominal tissue from birds of each diet group were used to prepare adipose explants. Tissue from three birds within a group was dissected to remove blood vessels and connective tissue, rinsed with phosphate buffered saline (PBS), and chopped into pieces of approximately 20 – 60 ng in size. Pieces were rinsed again with PBS and pooled within each diet group. Explants were distributed in two 96-well plates with approximately 100 ng of tissue per well. Wells contained Dulbecco's Modified Eagle's

Medium containing 5 mM glucose and 2% fatty acid free bovine serum albumin (ThermoFisher Scientific, Waltham, MA). Four hours after plating, media was changed and explants from each group were treated with 500 nM glucagon for two hours. After treatment, media was removed and non-esterified fatty acid concentration was measured using Wako Diagnostics' HR Series NEFA-HR (2) kit (Richmond, VA).

Statistical Analysis

Statistical analysis was completed using SAS 9.4 software (Cary, NC). The mixed model analysis of variance was analyzed using a completely randomized design with diet as the treatment factor. Results were considered statistically significant with a P value of less than 0.05. Significance levels and standard deviations for each set of data are represented within their respective figures.

3.4 Results

There was no significant difference in weight gain (P = 0.297, Figure 4.1), percent breast weight (P = 0.8644, Figure 4.2), or percent abdominal fat weight (P = 0.3281, Figure 4.3). There was also no difference in non-esterified fatty acid concentration, indicating that there was no difference in lipolysis activity (P = 0.114, Figure 4.4).

When average adipocyte size was analyzed, lard had a significantly larger size than all other diets with an average adipocyte size of 2038.12 μm^2 ± 48.60 (P < 0.0001, Figure 4.5). The coconut oil and macadamia oil diets were smaller than lard but could not be differentiated from one another with values of 1868.18 μm^2 ± 47.74 and 1858.73 μm^2 ± 46.15, respectively. Fish oil produced the next smallest adipocytes (1157.05 μm^2 ±

36.93), and tuna oil produced significantly smaller adipocytes than all other diets with an average size of 1035.18 μm^2 ± 34.85.

Smaller adipocytes tend to be more insulin sensitive, therefore the relative frequency of adipocyte size was calculated. The tuna oil group had significantly more adipocytes that were smaller than 1000 μm^2 with 57% ± 3.9 of adipocytes falling in this range ($P < 0.0001$, Figure 4.6), followed by fish oil with 44% ± 3.9. The remaining three diets all fell under 15%. In the range of 1000 – 1999 μm^2, the lard, coconut, and macadamia diets had significantly more adipocytes with 61%, 51%, and 47%, ± 4.7, respectively ($P = 0.0133$). Tuna oil had significantly fewer adipocytes in size range than all other diets, with only 35% ± 4.7 of cells being this size. Coconut oil (30% ± 2.8) and macadamia oil (31% ± 2.8) had significantly more adipocytes that fell into the range of 2000 – 2999, while fish oil (10% ± 2.8) and tuna oil (8% ± 2.8) had significantly less cells in this group ($P < 0.0001$). There was no difference between diets when adipocytes greater than 3,000 um^2 were counted ($P = 0.0918$).

When cell differentiation was investigated, macadamia oil and lard groups formed more lipid than tuna oil when the cells were allowed to differentiate spontaneously ($P < 0.0001$, Figure 4.7). In addition, the tuna oil group formed more lipid than the fish oil group. When differentiation was induced with fatty acids, all diets increased in lipid concentration. The macadamia oil diet had the greatest amount of lipid of all of the diets after treatment with the fatty acids.

Prior to treatment with glucagon, non-esterified fatty acid concentrations from the ex-vivo study showed that tuna oil had a greater concentration than fish and macadamia oils (P = 0.0491, Figure 4.8). After treatment with glucagon, tuna oil had a greater non-esterified fatty acid concentration than fish oil and macadamia oil, but could not be differentiated from coconut oil and lard (P = 0.0286). All diets had an increased non-esterified fatty acid concentration after exposure to glucagon.

Although there was no significant difference in PPARɣ expression between diets (P = 0.1313), there were some interesting trends between groups. Macadamia oil and tuna oil had the highest expression of PPARɣ, followed by coconut oil, fish oil, and finally lard with the lowest expression.

3.5 Discussion

As far as we are aware, this is the first study to look at diet as a means of reducing the likelihood of obesity in newly hatched chicks. In addition, we wanted to look at some factors related to obesity such as cellular differentiation into adipocytes, lipolysis, and expression of an obesity-related gene, PPARɣ.

Although no significant differences were reported for weight gain, percent breast weight, or percent abdominal fat weight, other unpublished thesis studies in our lab have found differences in these features in older chickens at approximately 30 days of age (Torchon, 2015). Therefore, we propose that the chicks were simply too young and too small to have shown statistically significant differences in these aspects of growth. However, we know that changes were being made at the cellular level due to

the different diets. There are two aspects of adipocytes that can be used to assess health status: hypertrophy and hyperplasia. Hypertrophy reflects an increase in the size of the cells, whereas hyperplasia is reflective of an increase in the number of cells. It has been established that the number of adipocytes in a human is determined early in development (Spalding et al., 2008), therefore it would be ideal to have measured adipocyte size with this study. Our study shows that there is the potential that factors such as diet can be used to manipulate the size of adipocytes, or hypertrophy, while an individual is very young. This could help increase profits for producers as trim loss has the potential to be reduced.

While nearly all diets were impacted by the fatty acids, some diets were affected more strongly than others when percent increase of oil red o absorbance was calculated. Macadamia oil treated with fatty acids increased absorbance by approximately 26% compared to the untreated, and tuna oil treated with fatty acids increased absorbance by approximately 43%. Fatty acid-treated fish oil experienced the most profound increase of 68%, indicating that it is most sensitive to the processes hyperplasia and/or hypertrophy. In addition, it is important to note that the fish oil treatment had significantly less lipid present during both spontaneous and induced differentiation. The lard and macadamia diets had the highest amount of lipid when they were left to spontaneous differentiation, but macadamia had significantly more lipid than all other diets when differentiation was induced.

Glucagon has been shown to stimulate lipolysis, or the breakdown and mobilization of lipids (Liljenquist et al., 1974). All diets treated with glucagon experienced an increase in non-esterified fatty acid levels, yet some diets were more sensitive to the treatment than other diets when percent increase was calculated. The fish and macadamia diets' non-esterified fatty acid concentrations increased by 70% and 101%, respectively, when treated with glucagon, whereas the coconut oil diet and tuna oil diet increased by approximately 50%. The lard diet showed only a 39% increase when treated with glucagon. This study demonstrated that diet can be used to make an individual's cells more sensitive to lipolysis, but it did not investigate apoptosis, or cell death. This is another element of release of fatty acids that could have been altered due to the diets, as it has been shown that DHA induces apoptosis in post-confluent preadipocytes in addition to promoting lipolysis in adipocytes in cell culture (Kim et al., 2006).

PPARɣ is a major regulator for adipogenesis, therefore it was one factor taken into consideration in this study. Although there was no significant difference in PPARɣ expression among diets, there were some interesting trends. Lard tended to have the highest expression and the fish oil diet seemed to have the lowest expression. This coincides with a study by Royan and others that showed that diets enriched in saturated fatty acids had higher PPARɣ expression in adipose tissue than chickens fed diets enriched in omega-3 fatty acids (Royan et al., 2011). The trend for increased PPARɣ in the saturated fatty acid based diets also supports our adipocyte size data, as PPARɣ

promotes lipid storage, and can thus increase the size of the adipocytes. Other transcription factors that may be of interest in the future for studies such as this include LXR (liver X receptor), HNF (hepatic nuclear factor), SREBP (sterol-regulating-element-binding protein), and AMPK (AMP-activated protein kinase) (Madsen et al., 2005).

This study was limited by the fact that it only focused on effects in adipose tissue. It is possible that there are changes in musculature (in particular, increase in breast weight). Fish oil has been shown to normalize lipid storage and glucose oxidation within the skeletal muscle (Lombardo et al., 2007). Perhaps there were significant changes happening in the body related to production and adiposity that were not covered by this study.

Overall, this study confirms that diet has the potential to impact incidence of childhood obesity at the cellular level. Further investigation of dietary programming could lead to more knowledge of the mechanisms of these effects and could help researchers find a better preventative approach to reduce the accumulation of excess adipose tissue in both chickens and humans.

3.6 Appendix: Figures and Tables

Table 3. Diet formulation

Feed Description	As-fed lbs. per 100 lb. bag
Corn meal	61.7
Soybean meal	32.2
DL Methionine	0.25
Salt	0.35
Limestone	1.10
Dicalcium phosphate	2.10
Vitamin Pre-Mix	0.25
Fat	1.90
Coban	0.05
Lysine	0.10

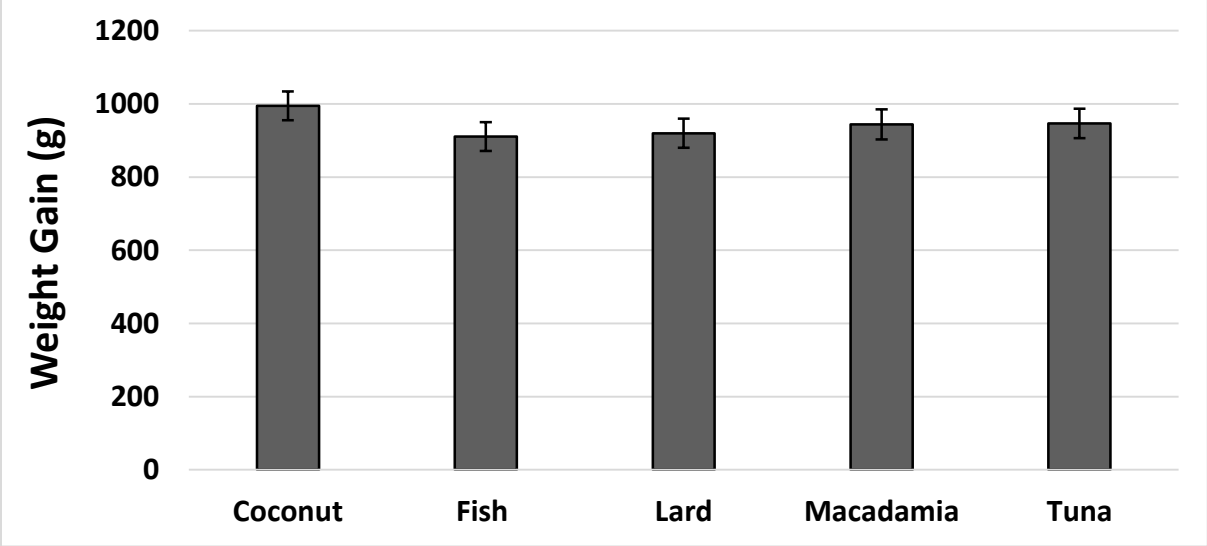

Figure 3.1. Comparison of weight gained across diets. Weight gain was calculated as final body weight at euthanasia minus body weight at hatch. Analysis of variance was performed (n = 87, P = 0.297).

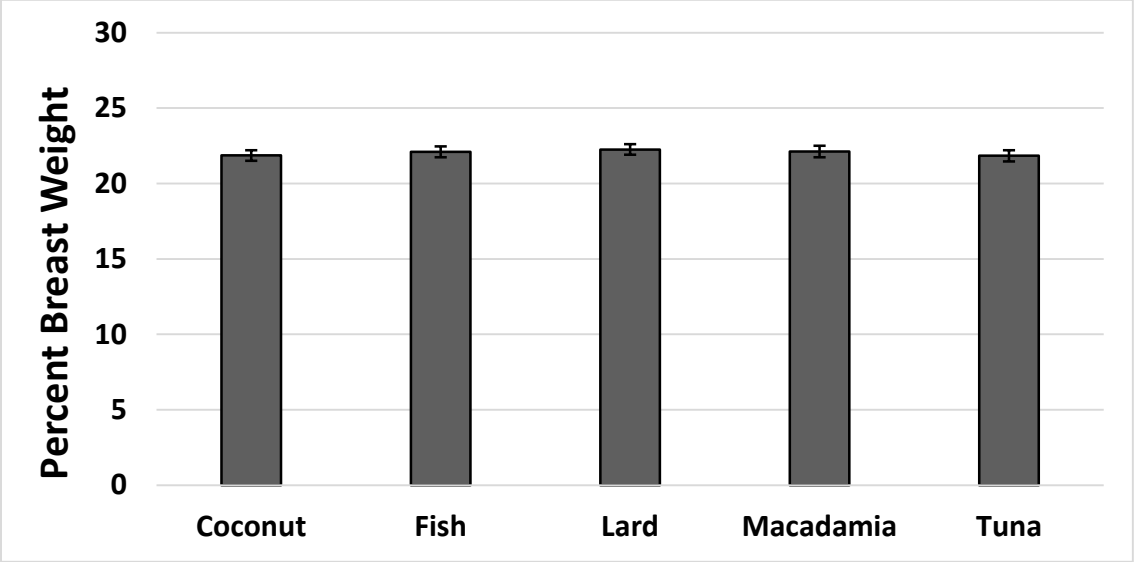

Figure 3.2. Percentage of breast muscle to final weight. This was calculated as breast weight divided by final body weight at euthanasia, converted to percentages. Analysis of variance was performed (n = 87, P = 0.8644)

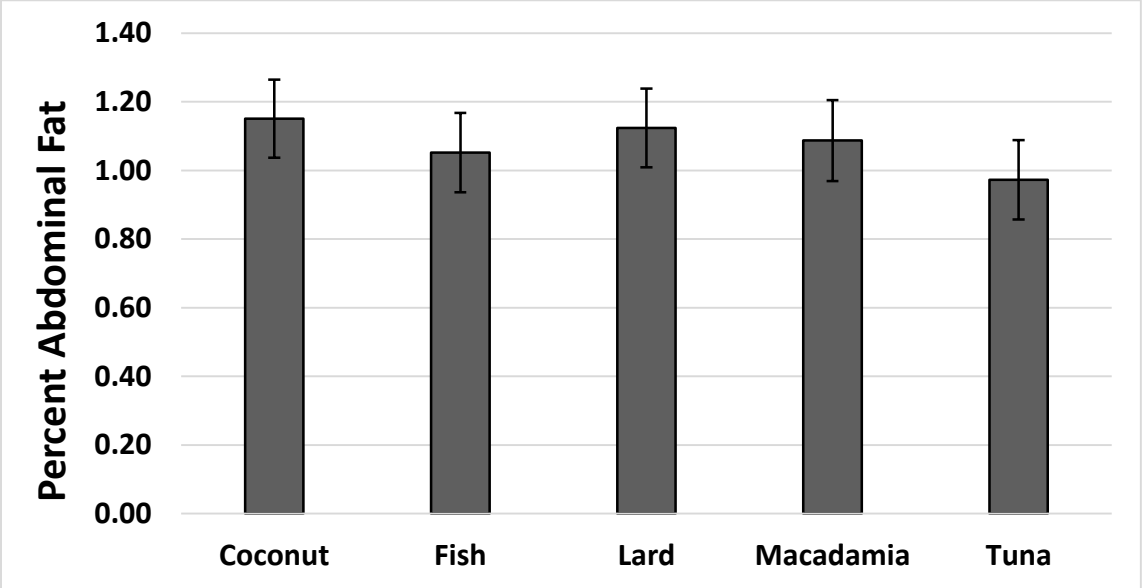

Figure 3.3. Percentage abdominal fat of final body weight. This was calculated as weight of the abdominal fat pad divided by the final body weight at euthanasia, converted to percentages. Analysis of variance was performed (n = 87, P = 0.3281).

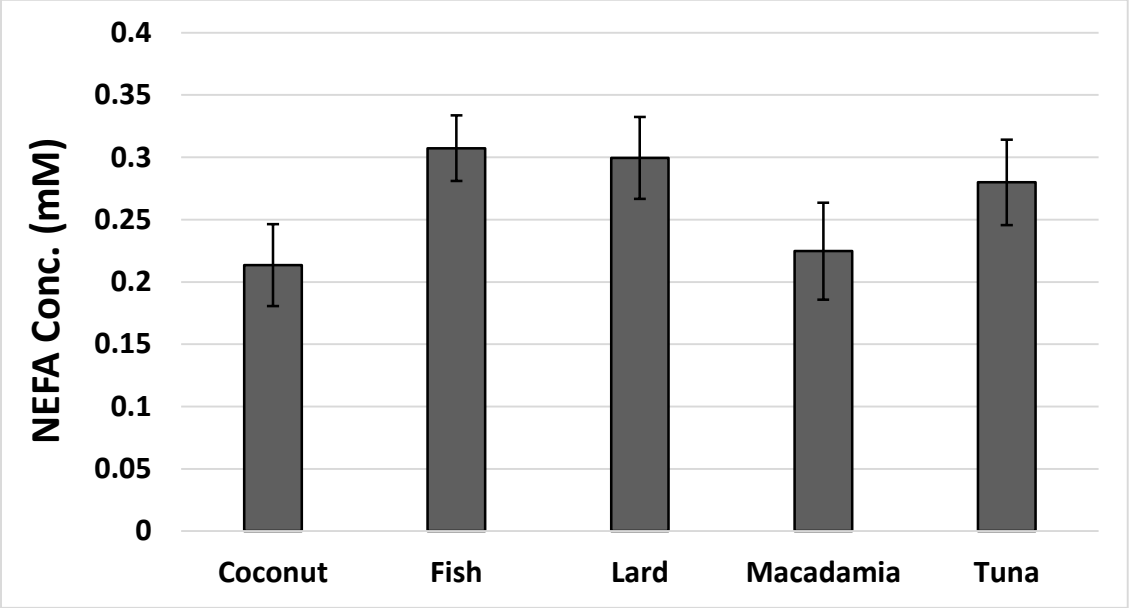

Figure 3.4. Non-esterified fatty acid concentrations. Analysis of variance was performed (n = 74, P = 0.114).

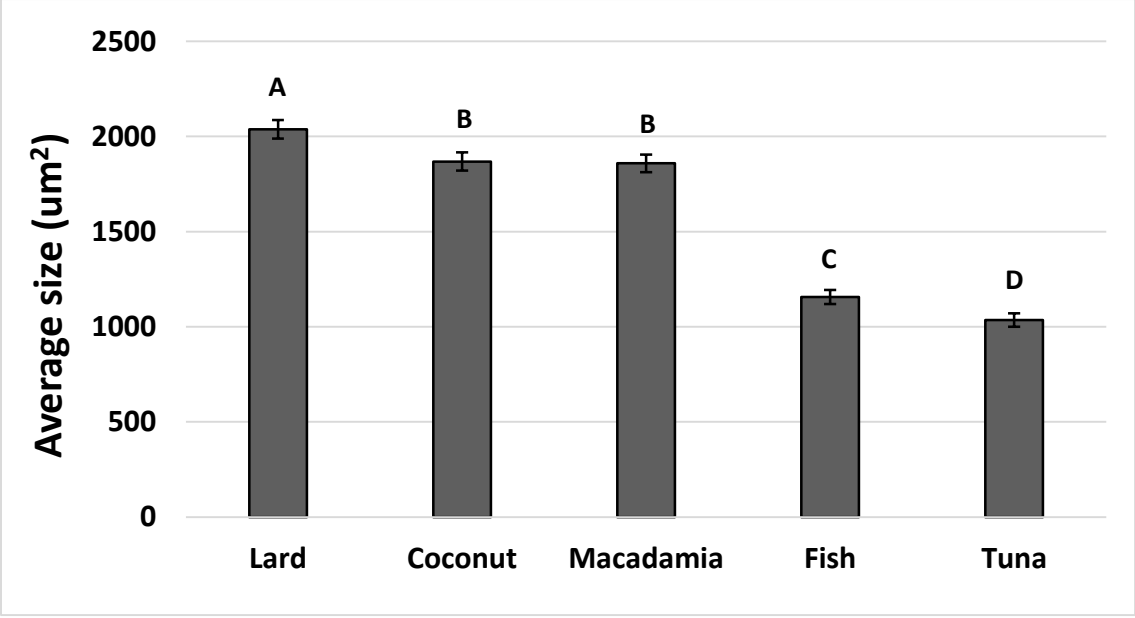

Figure 3.5. Comparison of average adipocyte size across diets. Analysis of variance was performed (n = 20, P < 0.0001).

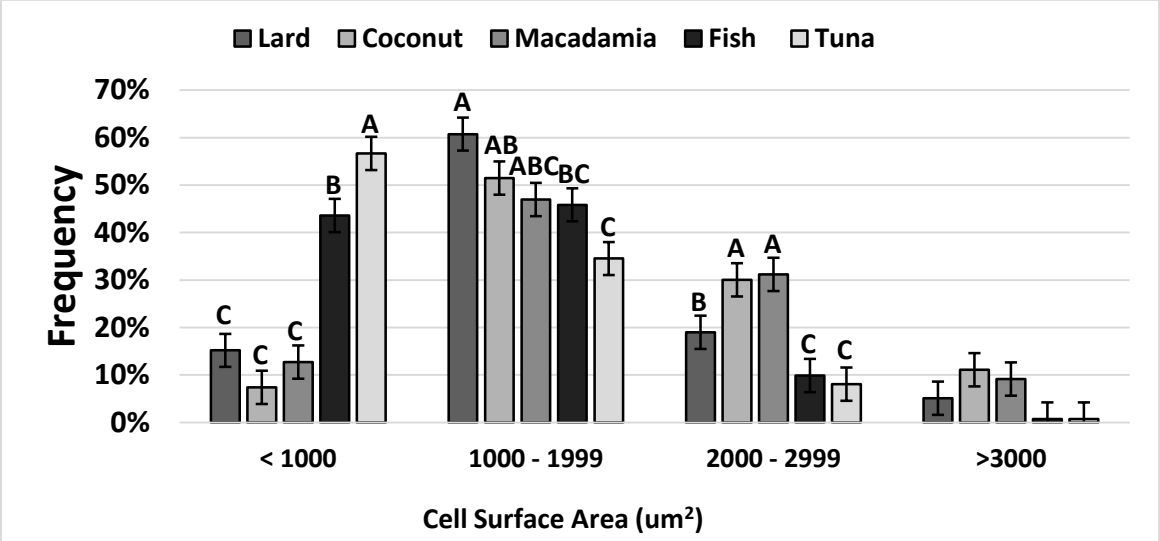

Figure 3.6. Relative frequency of adipocyte size (um^2). Analysis of variance was performed (n=20, P <0.0001, P = 0.0133, P < 0.0001, P = 0.09181, respectively). Letters are used to indicate statistically significant differences.

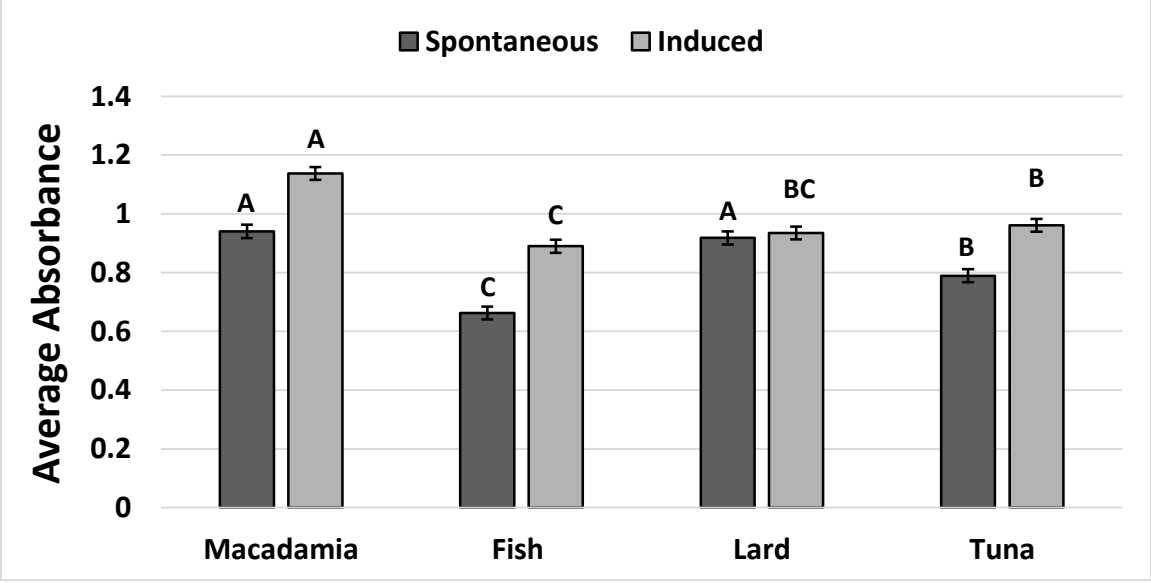

Figure 3.7. Average absorbance differentiation study. Analysis of variance was performed for the spontaneous and induced groups separately (P < 0.0001 for both groups). Letters are used to indicate statistically significant differences.

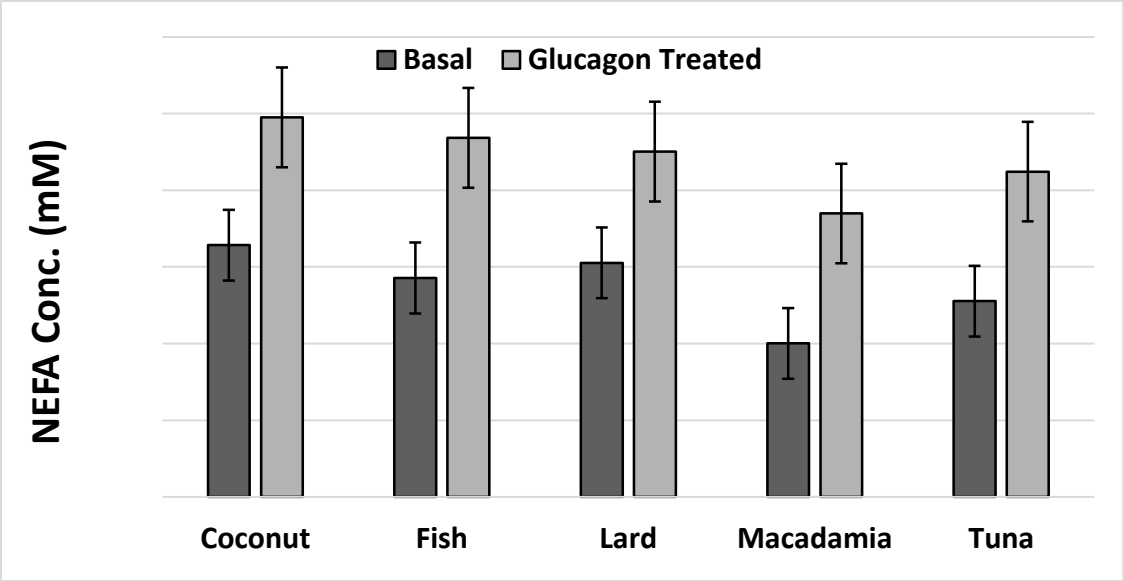

Figure 3.8. Basal vs. glucagon treated ex vivo non-esterified fatty acid concentrations. Analysis of variance was performed for basal and glucagon treated groups separately (P = 0.3360 and 0.7071, respectively).

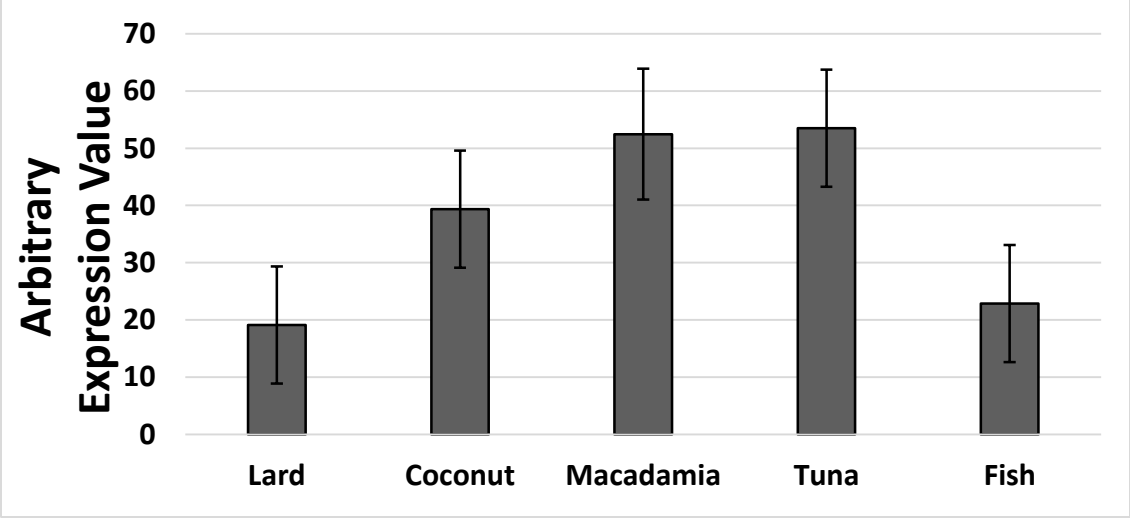

Figure 3.9. ΔCq was calculated as the average gene of interest (PPARɣ) expression minus the average housekeeping gene (TBC) expression. Analysis of variance was performed (P = 0.1313).

Chapter 4. In Ovo Supplementation of Omega-3 and Omega-6 Fatty Acids

4.1 Abstract

Excess fatness is a cause for profit loss in the broiler chicken industry, as fat has a much lower market value than muscle. *In ovo* injections have been used in broiler chickens to reduce adiposity, along with enhancing gut development and administering vaccines. Yet, there have been no known studies that investigate the effects of injecting fatty acids into the albumen on adiposity. This study was an attempt to enhance the understanding of the potential of using these injections to administer fatty acids. Forty-eight fertilized eggs were injected with an oil and egg white protein emulsification at day 17.5 of embryonic development. After hatching, the chicks displayed neurological symptoms that were ultimately unexplainable after necropsy. However, this study was the first step towards perfecting this technique for future investigation.

4.2 Introduction

The obesity problem in the United States is quickly reaching epidemic levels. In a survey taken in 2012, the Center for Disease Control found that over 30% of adults and nearly 20% of adolescents reported themselves as obese, or having a body mass index of greater than 30 (CDC, 2015). This excess fatness brings along many health-related issues including, but not limited to, cardiovascular disease, hypertension, diabetes, and cancer (Visscher & Seidell, 2001). On average, obese individuals pay an additional $1,429 per year in healthcare costs (Finkelstein et al., 2009). Children of obese parents are more likely to consume excess calories, and therefore are more likely to become obese themselves (Oliveria et al., 1992). Furthermore, children that are obese are more

likely to continue this trend into adulthood (Whitaker et al., 1997). Therefore, it is proposed that if obesity can be prevented in children, the cycle could be minimized altogether.

Humans are not the only animals that suffer from excess fatness. Broiler chickens have developed a propensity for excess fatness as producers have been breeding for larger birds to feed a growing population. Fat can cause a loss of profits as fat is generally trimmed off and discarded as a byproduct during processing. Broiler chickens are becoming increasingly popular as models for human obesity. Both humans and broilers rely on the liver for the majority of their lipid synthesis (Leveille et al., 1975). Both species also deposit much of their excess fat in the abdominal region. However, in chickens this fat pad does not begin to develop until after hatching, so it is a crucial point where adiposity could potentially be altered in growing offspring (Tzeng & Becker, 1981).

A prominent factor for the onset of obesity is the subject's diet. In particular, dietary fatty acids are a nutrient that is often looked at as a primary contributor. Omega-3 fatty acids have been associated with health benefits, including lessening the incidence of obesity (Ruzickova et al., 2004). There is potential for this fatty acid type to be given in the diet after hatch, or to be administered to the growing embryo while it is still developing in a process known as *in ovo* injections.

The *in ovo* method is currently used to administer certain vaccinations to the chicken embryo to improve immunity after hatching. It has also been shown to improve

the development of the gastrointestinal system when carbohydrates were supplemented in this way (Z. Uni et al., 2005). *In ovo* injections have also been shown to increase hatchability and decrease mortality (Z. Uni & Ferket, 2004). As far as is known, no research has been conducted to look at the effects of fatty acids being administered through *in ovo* injections.

4.3 Materials and Methods

Preliminary studies

To ensure that the planned *in ovo* injections were being administered in the appropriate location of the extra-embryonic cavity, injections were prepared using a 20% glycerol solution with bromophenol blue added in order to track the location of the injection. The broad side of fertilized broiler eggs at 17.5 days of embryonic development were cleaned using 70% ethanol and gauze. A Phillips head screwdriver was used to lightly drill a small hole in the top of the egg to remove the shell. One milliliter of the glycerol and bromophenol blue solution was drawn up into a 3 mL syringe with an 18 ga blunt-tipped needle. The needle was inserted approximately one inch and the injection was administered. Clear masking tape was placed over the site of injection. After 24 hours, the eggs were opened and the chicks were found to still be alive. They were euthanized via decapitation. It was noted that the injection could be found inside the amniotic sac and inside the liver and gastrointestinal tract, which was convincing evidence that the injections had been successfully placed without causing harm to the embryo.

Subject Selection and Assignment of Treatments

Forty-eight fertilized eggs were obtained from the Pilgrim's Pride poultry plant in Chattanooga, TN and were immediately placed into an incubator at 37.5˚C and 50% relative humidity. The eggs were evenly divided into one of two groups: corn oil or fish oil *in ovo* injections, and the shells were labeled with a permanent marker.

Preparation of Injections

At approximately 17.5 days of embryonic development, the injections were prepared. Because the inside of the egg is an aqueous environment, the decision was made to administer the injection in the form of an emulsification. First, 40mL of a 5% egg white powder solution was made using water. The mixture was placed on a stir plate with stir bar for approximately 20 minutes until it was mixed thoroughly. Then, 5mL of either sterilized fish oil or corn oil was added to 20mL of the egg white solution. The oil had been sterilized via vacuum filtration. The mixtures were homogenized at 10,000 RPM for 5 minutes. They were placed in the refrigerator for approximately 15 minutes so that the liquid could settle to the bottom and separate from the resulting foam.

Administration of In Ovo Injections

Following preparation of the injection, the eggs were sterilized with an alcohol prep wipe. One milliliter of the emulsification was drawn up into a 3 mL syringe and an 18 ga blunt-tipped needle was placed on the end. A Phillips head screwdriver was used to remove a small section of the broad end of the shell, just large enough for the syringe

to be inserted. The syringe was inserted through the first membrane, through the air sac, and through the other side of the air sac. A small amount of pressure was required to get through the second membrane. At that point, the injection was slowly and gently administered and the needle was slowly removed from the egg. A piece of clear masking tape was placed over the hole in the egg to prevent contamination from the environment.

Termination of Study

At approximately 21 days of embryonic development, the chicks hatched and were placed into one square foot pens with two birds per pen. Birds were fed a standard diet ad libitum, which is outlined in Table 4.

Table 4. Diet formulation

Feed Description	**As-fed lbs. per 100 lb. bag**
Corn meal	61.7
Soybean meal	32.2
DL Methionine	0.25
Salt	0.35
Limestone	1.10
Dicalcium phosphate	2.10
Vitamin Pre-Mix	0.25
Lard	1.90
Coban	0.05
Lysine	0.10

Unfortunately, within 48 hours, the majority of the chicks displayed symptoms of neurological issues. Their legs could not bear weight and they were unable to walk properly. Their hips presented in a splayed out manner and the chicks were unable to stand. The chicks that were displaying this behavior were euthanized immediately to prevent suffering. After losing all but six chickens, it was determined that the study should cease. This experiment was repeated with a new batch of eggs, but we experienced approximately the same mortality rate.

4.4 Discussion

Although this experiment did not produce the results that we had hoped for, much was learned from the experience. It is possible that our method of injection was not as sterile as anticipated. During normal development, the chick's first encounter with the world outside the eggshell is at hatch. This is when the chick is first exposed to microorganisms that could trigger an immune response. However, no matter how sterile we try to be, the use of *in ovo* injections are guaranteed to allow some contaminants into the chick's environment. After the first round of lipid *in ovo* injections, a chick was sent to the necropsy lab at the University Of Tennessee College Of Veterinary Medicine. The subsequent report indicated that the chicks may have been suffering from a bacterial infection of the gut. However, the second round of *in ovo* injections had the same mortality rate but with no indication of bacterial infection.

There is also a possibility that some sort of antigen was present in the oil that was injected, which would also trigger an immune response. At such a young age, the

chick would have no antibodies ready to bind to the antigen and neutralize whatever antigen caused the response. Perhaps our stimulation of the chick's immune system was simply too much for the embryo, and in turn the young chick, to handle.

Chapter 5. Conclusions

To conclude, the broiler chicken is a potentially valuable model for human obesity. In addition, the newly hatched broiler chick could serve as a model for childhood obesity in humans. The reduction in adipocyte size that was noted by enriching the diet in omega-3 fatty acids could increase insulin sensitivity and reduce the incidence of some obesity-related diseases in humans. Not only could this improve the health status, but it could also alleviate financial stress caused by the medical care needed to treat the obesity-related illnesses.

Although reducing caloric intake has been a common method for reducing obesity for many years, these studies investigated the potential of maintaining calories and only adjusting fat source in order to reduce obesity. Although there was no noticeable change in abdominal adiposity, weight gain and breast weight were maintained across diets, which is ideal for a poultry production setting where profit is based on musculature. These studies were limited by the fact that only abdominal fat was analyzed. It is possible that changes occurred in other tissues. If this is the case, it could open up doors for new products such as omega-3 fatty acid enriched meats, which could be an alternative way to increase profits for the poultry industry.

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Vita

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