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To the Graduate Council:

I am submitting herewith a dissertation written by Ronique Cleardo Beckford entitled "Unraveling the Molecular Mechanism of Fat Deposition Through Dietary Manipulation and Feed Restriction." I have examined the final electronic copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Animal Science.

Brynn H. Voy, Major Professor

We have read this dissertation and recommend its acceptance:

Jun Lin, John T. Mulliniks, Michael O. Smith, Margaret E. Staton

Accepted for the Council:

Dixie L. Thompson

Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records.)

# Unraveling the Molecular Mechanism of Fat Deposition Through Dietary Manipulation and Feed Restriction

A Dissertation Presented for the

Doctor of Philosophy

Degree

The University of Tennessee, Knoxville

Ronique Cleardo Beckford

December 2017

# DEDICATION

This dissertation is dedicated to my parents and family

## ACKNOWLEDGEMENTS

As a student, my success has been greatly influenced by a number of individuals in various ways. Therefore, it would be very remiss of me if I did not take the time out to show some appreciation to those who have contributed to my development and made my time at the University of Tennessee a success. First, I would like to give thanks to God for giving me this opportunity. Without him, my academic and personal pursuits and accomplishments would not have been possible.

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## ABSTRACT

This study was conducted to test the hypothesis that enriching developing adipose tissue in long chain omega-3 long chain polyunsaturated fatty acids (n-3 LCPUFA) reduces adiposity compared to enrichment in n-6 LCUFA. Sixty Cobb 500 broiler breeders were fed a diet containing either 5% fish oil (FO) or corn oil (CO). After four weeks, fertilized eggs were collected and hatched, and all chicks were fed the same corn oil-based broiler starter diet. At days 7 and 14 post-hatch, chicks in each group were weighed, euthanized and blood, liver, breast, and subcutaneous and abdominal fat samples harvested. Gas or liquid chromatography and mass spectrometry were used to determine the effects of diet on fatty acid profiles of tissue triglyceride, free fatty acid, phospholipid pools and protein expression. RNA sequencing and PCR was used to determine the effect on gene expression. Mixed model ANOVA and least square means were used to identify effects of maternal diet and age*diet interactions on chick phenotypes (SAS v9.4, Cary NC). Differentially expressed genes were determined using the DESeq2 R package. Hen dietary fat source did not significantly affect performance. Phospholipids and total lipids in muscle and adipose tissue of FO-chicks were significantly enriched in eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) species relative to CO-chicks, with corresponding reductions in n-6 LCPUFA ($p<0.05$). FO-chicks had significantly lower abdominal and subcutaneous fat deposition ($p<0.05$). Proteomics identified 95 known proteins that differed in abundance between FO and CO adipose tissue. KEGG pathway enrichment analysis of differentially abundant proteins indicated that hen FO feeding significantly suppressed glycolysis (n=5 proteins) and altered the cytoskeletal architecture of adipocytes and lipid droplet proteins (n=11 proteins). Hen FO resulted in 190 upregulated and 46 downregulated genes 7 days and 13 upregulated and 32 down regulated genes at 14 days Combined, these results suggest that hen FO programs reduced adiposity by promoting

differentiation of adipocytes with reduced capacity for fatty acid uptake and storage. Future studies are needed to identify long term effects of this method of programming and additional strategies that may be necessary to sustain its effect.

**TABLE OF CONTENTS**

CHAPTER I. LITERATURE REVIEW ..... 1

Excess fat accumulation in humans and animals ..... 1

Broilers as a model for understanding excess fat deposition ..... 2

Developmental programming of obesity ..... 4

Polyunsaturated fatty acids ..... 5

Epigenetic Regulation of gene expression ..... 7

References ..... 9

CHAPTER II. DIETARY FAT SOURCES, ADIPOSITY AND PRODUCTION TRAITS IN POULTRY ..... 14

Abstract ..... 15

Introduction ..... 15

Dietary fat types included in poultry diets ..... 18

Impact of dietary fat type on performance and bird health ..... 18

Influence of dietary fat type on fatty acid profile and quality of meat and egg ..... 20

Influence of dietary fat source on consumer perception and health ..... 21

Summary and conclusion ..... 21

References ..... 23

CHAPTER III. ENRICHING THE HEN DIET IN LONG CHAIN N-3 POLYUNSATURATED FATTY ACIDS PROGRAMS CHICKS TOWARDS REDUCED ADIPOSITY ..... 26

Abstract ..... 27

Introduction ..... 28

Materials and Methods ..... 30

Diets and husbandry ..... 30

Blood and tissue collection ..... 31

Serum metabolites ..... 31

Fatty acid analysis ..... 31

Phospholipid analysis ........ 32
Adipocyte size ........ 32
Real time PCR assay ........ 33
Proteomics ........ 33
Statistical Analysis ........ 34
Results ........ 34
Egg production and chick fatty acid composition ........ 34
Body weight and adipose deposition ........ 35
Quantitative Polymerase Chain Reaction ........ 36
Adipose tissue proteomics ........ 37
Discussion ........ 38
References ........ 44
Appendix ........ 50
CHAPTER IV. TRANSCRIPTIONAL ANALYSIS OF ABDOMINAL FAT OF BROILER CHICKS PROGRAMMED TOWARDS REDUCED FAT DEPOSITION ........ 62
Abstract ........ 63
Introduction ........ 64
Materials and Methods ........ 66
Tissue collection and RNA extraction ........ 66
RNA sequencing ........ 66
Trimming and mapping ........ 67
Differential gene expression ........ 67
Results ........ 67
Discussion ........ 69
References ........ 72
Appendix ........ 75

CHAPTER V. TRANSCRIPTOMIC AND METABOLOMIC PROFILING OF CHICKEN ADIPOSE TISSUE: DUAL PURPOSE BENEFIT FOR HUMAN OBESITY AND POULTRY PRODUCTION ..... 84
Abstract ..... 85
Introduction ..... 85
Adipose tissue development ..... 86
Models of differential fatness ..... 88
Omics approaches to understand adipose deposition ..... 88
Insight into control of adipose tissue growth and metabolism in chickens from transcriptomics and metabolomics ..... 92
Conclusions and future directions ..... 98
References ..... 99
Appendix ..... 104
CHAPTER VI. CONCLUSION AND FUTURE DIRECTIONS ..... 105
APPENDIX Enriching the starter diet in Omega 3 polyunsaturated fatty acids reduces adipocyte size in broiler chicks ..... 107
Abstract ..... 108
Introduction ..... 109
Materials and Methods ..... 110
Animals and diets ..... 110
Statistical analysis ..... 110
Results ..... 111
Effects on body composition and growth rate ..... 111
Effects on Relative mRNA Expression in Visceral White Adipose Tissue and Liver ..... 111
Discussion ..... 112
References ..... 116
Appendix ..... 119
Supplemental Methods ..... 122

VITA .......................................................................................................................... 128

## LIST OF TABLES

Table 3.1. Composition of FO and CO broiler breeder diets fed to hens for 28 d............50

Table 3.2. Enrichment of brain and liver phospholipids at hatch in broiler chicks produced from hens fed diets containing FO or CO for 28 d..................................................................51

Table 3.3. Abdominal adipose tissue fatty acid content of broiler chicks produced from hens fed diets containing FO or CO for 28 d ........................................................................52

Table 3.4. Effect of dietary enrichment on performance and serum metabolites of broiler chicks produced from hens fed diets containing FO or CO for 28 d ..........................................54

Table 3.5. Differentially expressed proteins in abdominal adipose tissue of FO vs. CO chicks at 14 d ..........................................................................................................................55

Table 3.6. Functional enrichment of differentially abundant proteins in adipose tissue of chicks produced from hens fed diets containing FO or CO for 28 d ..........................................58

Table 4.1. Summary of reads mapped from RNA Sequencing of abdominal adipose tissue of FO and CO chick at 7 and 14 d...........................................................................................75

Table 4.2. Genes upregulated in abdominal adipose tissue of FO vs. CO chicks at 7 d...76

Table 4.3. Genes downregulated in abdominal adipose tissue of FO vs. CO chicks at 7 d ..........................................................................................................................80

Table 4.4. Genes upregulated in abdominal adipose tissue of FO vs. CO chicks at 14 d.81

Table 4.5. Genes downregulated in abdominal adipose tissue of FO vs. CO chicks at 14d ..........................................................................................................................82

Table 1. Effects of dietary LA, CA, FL and FO on body, adipose and muscle weights and on serum metabolites in broiler chicks[1] ........................................................................119

Table S.1. Base experimental diet composition..............................................................125

Table S.2. Effects of dietary LA, CA, FL and FO on fatty acid profiles of phospholipids and total lipids in abdominal adipose tissue[1] ........................................................................126

## LIST OF FIGURES

Figure 3.1. Adipocyte volume and number in CO and FO chicks at 7 and 14 d of age. Representative H&E-stained images of abdominal adipose tissue from CO (A, B) and FO (C, D) used to determine adipocyte volume at 7 (A, C) and 14 (B, D) d. Scale bar = 40 µm. Two slides and three independent fields/slide were counted in three chicks in each age/diet group. (E), average adipocyte volume ($\mu m^3$ X $10^4$), ± SD; (F) average adipocyte number (X $10^6$),± SD; * = P<0.05, FO vs. CO, post-hoc comparison using LSM ....................................................59

Figure 3.2 Frequency distribution of adipocyte area ($\mu m^2$) of CO and FO chicks at 7 (A) and 14 (B) d of age. Adipocyte areas measured from H&E-stained images were divided into arbitrary bins; average frequency of cells within each bin, ± SEM; * = P $\leq$ 0.05; # = P $\leq$ 0.10, FO vs. CO. ........................................................................................................................60

Figure 3.3. Expression of genes involved in adipogenesis, fatty acid oxidation and uptake of fatty acids in adipose tissue (A, B) and liver (C) of CO and FO at two weeks of age.ACOX1, Acyl-CoA oxidase 1; CPT1, Carnitine palmitoyltransferase 1; PPARG, Peroxisome proliferator-activated receptor gamma; PPARGC1B, Peroxisome proliferator-activated receptor gamma co-activator 1 Beta; FASN, Fatty acid synthase; LPL, Lipoprotein lipase; average relative abundance, ± SEM;* = P $\leq$ 0.05, FO vs. CO; n=5-6/diet group......................................61

Figure 4.1. Hierarchical clustering separates chicks according to diet and age. The 50 most variable genes between the four groups were analyzed using Pearson's correlation. Distance between samples and genes were determined using complete linkage............................83

Figure 5.1. Similarities in adipose tissue gene expression profiles between genetically-driven leanness and fasting. Unsupervised clustering was used to visualize similarities in gene expression profiles from abdominal adipose tissue of fed and fasted (five hours) 21 day-old broiler chicks (Ji,

et al., 2012), and from fed adult broilers and genetically lean Fayoumi chickens (Ji, et al 2014). Expression profiles from fasted broiler chicks were more similar to those of the genetically distinct, lean Fayoumi line than to profiles from chick or adult broilers that were fed.................104

Figure 1. Effects of dietary FO, FL, CA and LA on abdominal adipocyte size distribution and on gene expression in liver and adipose tissue. (A), Adipocyte area was measured from images of H&E-stained sections of abdominal adipose tissue using Image J (Version 1.48, National Institutes of Health); areas < 500 $\mu m^2$ were removed from analyses. Adipocyte volume ($\mu m^3$) was calculated from area ($\mu m^2$). Frequency distributions were produced by grouping adipocytes into bins based on volume and counting the frequency of cells within each bin. ANOVA was used to evaluate the effect of diet within each bin. Values with different superscripts differ significantly ($P < 0.05$) based on post-hoc testing when ANOVA indicated a significant effect of diet ($P < 0.05$); (B, C). Relative mRNA expression of genes involved in lipid metabolism, adipogenesis and inflammation in abdominal adipose tissue (B), and in lipid metabolism in liver (C) , N=6 birds / diet. Values are group means ± group std. dev.; values with different superscripts differ significantly ($P < 0.05$) based on post-hoc testing when ANOVA indicated a significant effect of diet ($P < 0.05$); Lard (LA), Canola Oil (CA), Flaxseed oil (FL), Fish oil (FO). ..............................................120

Figure S.1. Representative H&E-stained images of abdominal adipocytes; (A) Lard (LA), (B) Canola Oil (CA), (C) Flaxseed oil (FL), (D) Fish oil (FO); 20X magnification. .............127

# CHAPTER I.

# LITERATURE REVIEW

## Excess fat accumulation in humans and animals

Humans and other animal species store energy in the form of fat in order to maintain energy homeostasis. When energy intake is low, fat is mobilized and oxidized to provide the organs with energy. However, in some cases, the stored energy is not always utilized, thereby resulting in high accumulation of fat. If this excess energy is not utilized, over time, there are various negative consequences that can occur both in humans and in animals. In humans, the abnormal or excessive accumulation of fat, referred to as obesity, results in impaired health and an increased risk of metabolic and cardiovascular diseases (Buckley, et al., 2009; Chang, et al., 2015). Based on data collected in 106 countries, Kelly, et al., (2008) projected that the number of obese individuals are expected to increase and by the year 2030 there could be approximately 2.16 billion overweight and 1.2 billion obese individuals living in the world. Currently, in the United states, more than one third of adults and 17% of children are considered obese (Ogden, et al., 2014). If this trend continues there will be serious implications for public health and potentially an epidemic. Various factors have been implicated in the cause of obesity including genetics, high energy diets and low levels of physical activity (Rippe, et al., 1998). Combined, they make it very difficult to treat obesity hence more effort needs to be placed on prevention (Hill, et al., 1998). However, in order for prevention to be effective an understanding of the molecular mechanisms that regulate fat deposition and how they can be altered is necessary. Over the years, rodents have been the model utilized to gain insight into the mechanisms that result in fattening in mammals including humans. However, in recent years, chickens, particularly broilers (birds reared for meat) have been gaining more prominence as a model organism for obesity.

In chickens, excess fat accumulation is also a problem. In order to supply the growing demand for chicken and chicken products, poultry breeders selected and bred various breeds of chicken to develop strains of broilers that have increased growth and feed efficiency. However, one negative consequence of this selection of broilers for rapid growth is an increase in fat content of the marketable product (Griffin, et al., 1994) and an increase in fat accumulation in the abdomen (Fouad, et al., 2014). Though the fat deposited in the meat adds flavor and may influence consumers' perception, abdominal fat deposition essentially wastes feed, increases processing costs and nitrogen waste, and is a negative trait to consumers who are conscious of their dietary fat intake (Ji, et al., 2012). In addition to broilers, excess fat is a concern in layers and broiler-breeders because it impairs fertility and immune function (Siegel, et al., 2003). In addition, as with human obesity, excess fat may also impair skeletal muscle energy metabolism and compromise the efficient growth of lean tissue (Kelley, 2002).

**Broilers as a model for understanding excess fat deposition**

Through years of breeding and selection, broilers have been developed for rapid growth, and high feed efficiency. Being confined, they are not required to exert much energy to find food or participate in other natural activities. In addition to that, the diet they consume is high in energy. Similar to an obese human, most of the excess fat is stored in their abdomen. Some studies have shown that the molecular mechanisms regulating fat deposition in broilers and humans are very similar, making broilers a good model for understanding fatness in humans (Resnyk, et al., 2013). Also, like humans, the primary place of de novo lipogenesis is the liver, and very low density lipoprotein is responsible for the transport of fatty acids from the liver to the depots (Saadoun, et al., 1987). Therefore, taking advantage of these similarities, various research groups, including ours, have been utilizing broiler chickens as a model to better understand the mechanisms that

drive fat deposition and identify ways to regulate this process (Ji, et al., 2014). Though the development of chicks takes place outside of the hen, the chicken-egg relationship provides researchers with a model that allows for testing the direct impact of early exposure to certain nutrients. For example, by feeding hens diets enriched in various fatty acids, Koppenol, et al., (2015) established that fatty acids were taken up by the yolk and passed on to the offspring via the yolk sac. They concluded that, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) were incorporated into the yolk sac and transferred to the developing embryo. They also noted that at 28 d hepatic EPA and DHA concentrations were only affected in the perinatal period. Ji, et al., (2012) further highlighted the usefulness of chickens as a model by identifying pathways through which fat accumulation may be reduced by using genetic selection and management practices such as fasting. In an effort to understand the effect of the synthetic glucocorticoid dexamethasone on lipid metabolism, with broilers as a model, Cai, et al., (2009) reported that dexamethasone enhanced lipid metabolism. They were able to identify various genes involved in lipid synthesis, uptake and utilization that were significantly altered. By feeding diets that varied in the fatty acid profile, Crespo, et al., (2001) concluded that the fatty acid profile of various tissues was a direct reflection of dietary fatty acid intake. In addition they concluded that diets containing polyunsaturated fatty acids decreased abdominal fat compared to saturated or monounsaturated fatty acids. Therefore, this relationship provides a unique model to study the role of nutrition in the maternal-fetal system because during development the embryo is in an isolated environment not under the influence of nutrients from maternal circulation as in the mammalian system (Cherian, 2008).

**Developmental programming of obesity**

Developmental programming refers to the concept that changes in the environment within critical periods of development such as gestation or lactation has a permanent impact on metabolism and can result in the onset of certain diseases in adulthood. Since the period from conception to birth is a time of rapid growth, cellular differentiation and replication, exposure of the developing embryo to certain chemicals can have a long term impact on development (Simmons, 2008). Studies suggest that macronutrient intake influenced by maternal diet and body composition likely plays a role in the metabolic conditioning of the fetus in utero (Elshenawy, et al., 2016). These variations are based on epigenetic changes that alter the expression of various genes (Lisboa, et al., 2012). Different environmental stimuli which include diet, lifestyle habits (smoking, drinking) and other chemical exposures have been implicated in causing epigenetic changes. For example, Veiga-Lopez, et al., (2016) reported that exposure to Bisphenol A (BPA) caused adipocyte hypertrophy in the visceral fat without an increase in the fat mass. These authors also concluded that exposure to BPA during fetal development at levels found in humans can increase the potential for insulin resistance. Similarly, Timmermans, et al., (2014) found that maternal smoking resulted in lower birth weight and greater body weight gain in the first year of life and increased body weight gain during infancy.

Diet has also been shown to have a programming effect. Both diets low and high in various nutrients can have this impact. Saad, et al., (2016) reported that pups from dams fed a high fructose diet had a higher risk of obesity, hypertension, and metabolic dysfunctions. In sheep, maternal obesity increased fetal adiposity, fatty acid and glucose transporters, and expression of enzymes involved in fatty acid biosynthesis (Long, et al., 2012). According to Campbell, (2012), the inclusion of functional foods such as omega 3 fatty acids may be an approach to aid in the

management of obesity. In a recent study in humans, Vidakovic, et al., (2016) reported lower maternal n–3 PUFA concentrations and greater n–6 PUFA concentrations during pregnancy are associated with greater body fat and abdominal fat in childhood. Similarly, women consuming fish diets high in omega 3 polyunsaturated fatty acids at least three times per week had children with increased body mass index, growth, and adiposity in the first six years of life (Stratakis, et al., 2016).

**Polyunsaturated fatty acids**

According to Madsen, et al., (2005), far more attention has been focused on strategies for treating obesity-associated diseases rather than focusing on preventing or treating the major underlying risk factor, obesity. They further stated that, understanding the processes that lead to de novo differentiation of adipocytes and onset of obesity would be necessary for developing new rational modalities for the prevention and treatment of obesity. Apart from their role as storage and transport forms of metabolic fuel, the fatty acid portion of dietary fats serves several other important functions in the body. These include providing for the essential fatty acid requirements, acting as structural components of cell membranes and serving as precursors for eicosanoid production (Fritsche, et al., 1993). Fatty acids are classified based on their length, location and number of double bonds they contain in relation to the methyl end of the fatty acid chain. These characteristics determine their specific activity and metabolic characteristics (Madsen, et al., 2005). Saturated fatty acids contain no multiple bonds while unsaturated fatty acids contain one or more multiple bonds. Polyunsaturated fatty acids are classified as n-3 or n-6 based on the location of the first double bond from the methyl end of the carbon chain (Schmitz, et al., 2008).

Research in both human and animal models has shown that n-3 fatty acids have distinct and important bioactive properties compared with other groups of fatty acids. In vitro studies with

adipocyte cell lines demonstrate that in comparison to their n-6 counterparts, n-3 fatty acids differentially regulate preadipocyte proliferation, adipogenesis, and triglyceride storage. Omega-6 PUFAs, particularly arachidonic acid (AA; 20:4 n-6) tend to be pro-adipogenic (Gaillard, et al., 1989; Massiera, et al., 2003; Negrel, et al., 1989), while LC n-3 PUFAs EPA (20:5 n-3) and (DHA; (22:6 n-3) attenuate lipid accumulation and promote an oxidative adipocyte phenotype (Fleckenstein-Elsen, et al., 2016; Kim, et al., 2006; Manickam, et al., 2010). In addition, n-3 fatty acids are known to reduce many risk factors associated with several diseases, such as cardiovascular diseases, diabetes, and cancer (Deckelbaum, et al., 2006).

The simplest member of the omega-3 family, alpha-linolenic acid (18:3 n-3), can be converted to the more long chain n-3 PUFA and is considered to be the only dietary essential n-3 PUFA. It can be converted into biologically active very long-chain n-3 PUFA, EPA and DHA through a series of desaturation and elongation reactions. However, this process is not very efficient therefore, EPA and DHA are normally required in the diet (Wojcik, et al., 2014). Long-chain n-3 PUFA have a range of physiological roles that relate to optimal cell membrane structure and cell function and responses. Their longer chain length, high number of double bonds, and presence of the first double bonds enables them to generate different metabolites from other polyunsaturated fatty acids (Deckelbaum, et al., 2006).Thus, they may play key roles in preventing, and perhaps treating, many conditions of poor health and well-being. The multiple actions of n-3 PUFA appear to involve multiple mechanisms that connect the cell membrane, the cytosol, and the nucleus. For some actions, n-3 PUFA appear to act via receptors or sensors, so regulating signaling processes that influence patterns of gene expression (Calder, 2012).

**Epigenetic Regulation of gene expression**

DNA methylation is a class of epigenetic regulation in which a cytosine base is modified by DNA methyltransferase at the C5 position of cytosine. Approximately 70% of CpG (5'-cytosine phosphate-guanosine) dinucleotides in human DNA are constitutively methylated, whereas most of the unmethylated CpGs are located in CpG islands. CpG islands are CG-rich sequences located near coding sequences and they serve as promoters for their associated genes (Elshenawy, et al., 2016; Hoile, et al., 2013). The methylation status of CpG islands within promoter sequences works as an essential regulatory element by modifying the binding affinity of transcription factors to DNA binding sites. In normal cells, most CpG islands remain unmethylated; however, under circumstances such as oxidative stress, they can become methylated de novo. Methylation is accompanied by changes in histone modification and chromatin structure, such that the CpG Island and its embedded promoter take on a repressed conformation that is incompatible with gene transcription (Elshenawy, et al., 2016). One critical gene involved in lipid metabolism that has been shown to be affected by methylation is the peroxisome proliferator activating receptor (PPAR) (Bagi, et al., 2004).

Peroxisome proliferator activating receptors are transcription factors that regulate gene expression and so have a role in cell and tissue responses to the environment (Yoon, 2009). PPAR-alpha (PPARα) and PPAR-gamma (PPARγ) are the most well understood PPAR isoforms. PPARα is expressed mainly in the liver. It is involved in regulating hepatic responses to the availability of certain fatty acids, fatty acid metabolites, and other peroxisome proliferators. PPAR-gamma is expressed in adipose tissue, where it regulates adipocyte differentiation and regulates the metabolic responses of adipocytes, including promoting insulin sensitivity. Two isoforms of PPARγ are formed by alternative splicing, of the two, PPARγ2 is predominantly found in adipocytes (Wu, et

al., 1999). During adipogenesis, PPARγ plays a critical role in the commitment of fibroblasts to become adipocytes in a ligand dependent way (Hihi, et al., 2002). Though other transcription factors may participate in adipogenesis, PPARγ is considered to be the only one necessary (White, et al., 2010). According to Rosen, et al., (2000) transcriptional regulators of adipogenesis such a PPAR tend to operate in a feed forward manner by inducing other pro-adipogenic factors and then working with those factors to increase gene expression.

Firstly, they form a heterodimer with the retinoic-X-receptor (RXR) and upon binding interact with cofactors such that the transcription initiation is increased (Berger, et al., 2002; Wu, et al., 1999). This effect is elicited by the heterodimer binding to a short sequence of DNA, the PPAR response element in the promoter region of target genes (Bordoni, et al., 2006). Secondly, ligands binding to PPARs cause it to be activated and target various genes that play a role in adipogenesis, and lipid and glucose metabolism (Ryan, et al., 2011). Therefore, despite structural differences, PPARs are activated by noncovalent binding of ligands that include n-3 PUFA and various eicosanoid mediators (Calder, 2012) and serve as lipid sensors which when activated, significantly change metabolism (Evans, et al., 2004).

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# CHAPTER II.

# DIETARY FAT SOURCES, ADIPOSITY AND PRODUCTION TRAITS IN POULTRY

A version of this chapter is being prepared for publication as a review article by: Ronique C. Beckford, Robert I. Mihelic, Michael O. Smith, and Brynn H. Voy

**Abstract**

The primary goal of poultry producers is to meet the market demand for meat and eggs. While being efficient, poultry producers must ensure the production of a healthy, wholesome product that is beneficial to the consumers' health. During diet formulation, fat is used to ensure that the birds' energy requirement is met. However, dietary fatty acids also have a direct impact on the health of the birds, meat and egg quality, and fatty acid profile. Ultimately, consumers eating eggs and meat will be directly impacted by the fatty acids supplied by these products. In recent years, dietary fatty acids have been receiving much attention and various recommendations have been made about their daily intake in humans. Some fatty acids have been linked to various diseases and have been found to have adverse health effects. For example, consumption of diets that are low in omega-3 and high in omega-6 fatty acids have an increased risk of suffering from various illnesses such as obesity, cardiovascular diseases, and cancer in humans. Therefore, when considering the type of fat to include in the birds' diet these factors should be taken into consideration. This review will highlight various types of fat that have been utilized in the diet of layers and broilers and the impact they have on performance, health status, fat deposition, product quality, as well as on consumer preference and health.

**Introduction**

The poultry industry is very dynamic and has responded to consumer demand in numerous ways. In an effort to adapt to consumer demands, adjustments have been made in management and production in the poultry industry. Consequently, in recent years there has been an increase in organic, no hormone added, no antibiotic ever, and cage free egg and meat production. Despite these changes, not much has been done in the area of meat and egg enrichment. Various studies

have shown that the diet consumed by broilers and layers has a direct impact on the fatty acid content of their products. Unlike in ruminants where ruminal microbes alter dietary fatty acids and change the fatty acids deposited in lean tissue, in poultry the fatty acid content of the meat and egg is a direct reflection of the diet (Woods, et al., 2009). Therefore, manipulating the chickens' diet by adding feed ingredients that provide essential nutrients to consumers may be an avenue to improve the essential nutrient dietary intake. One such nutrient that is not consumed at the recommended amounts is omega-3 polyunsaturated fatty acid (n-3 PUFA). In particular, the long chain n-3 PUFA eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), which have been proven to have numerous salubrious properties. However, with consumer awareness of the health benefits of these fatty acids increasing, there is a demand for enriching foods in with n- 3 fatty acids providing an opportunity for poultry producers to add value to their product (Zuidhof, et al., 2009).

Long chain n-3 fatty acids are considered dietary essential due to the low rate at which they are synthesized from precursors. In the human diet, fish has long been the source of EPA and DHA. However, over the past 100 years, there has been major changes in the type of fish consumed (Hulan, et al., 1988) and an increase in other protein sources that are high in n-6 fatty acids. High levels of n-6 fatty acids in the diets have been associated with a number of chronic diseases. Compared to n-6 fatty acids, n-3 fatty acids decreases the occurrence of cardiovascular related diseases (Calder, 2012) cancer, osteoporosis, and inflammatory autoimmune diseases (González-Esquerra, et al., 2001; Simopoulos, 2006). Currently, the world health and American heart organization recommends the consumption of 200 to 500 mg of n-3 PUFA in the form of EPA and DHA 1 to 2 times per week for cardiovascular health (Campbell, 2012). However, daily intake especially in the western hemisphere fall below the suggested amounts (Cherian, 2008b).

Adding fat to poultry diets is a common practice which is usually done to provide a dense energy source, control dust, improve diet palatability and provide essential fatty acids (Azain, 2004). In addition, there is a strong relationship between dietary fatty acids and the fatty acids of the end products of non-ruminants (Kouba, et al., 2011) which has been used to produce n-3 fatty acid enriched meat and eggs. In addition, studies have used it to directly alter the nutritional content of eggs to provide the developing embryo with essential fatty acids during development (Cherian, 2008a). Other studies have reported a reduction in the n-6 to n-3 fatty acid ratio in the meat of broilers fed ingredients high in n-3 fatty acids (Hulan, et al., 1988; Rahimi, et al., 2011). Furthermore, studies have also reported that inclusion of n-3 fatty acids to the diet reduces the accumulation of abdominal fat and helps to alleviate the negative consequences of excess fat deposition.

With poultry products being the number one source of protein in western diets and most countries around the world, enriching poultry products may be a viable option to increase the amount of n-3 fatty acids in the human diet. Over the years, various approaches have been taken to evaluate the differential effects of dietary fat types in poultry diet. Despite the number of studies conducted, no consensus has been reached for required levels of n-3 inclusion nor has a suitable inexpensive source been identified. In addition, inclusion of high levels of n-3 polyunsaturated fatty acids may have detrimental effects on immune function in chickens (Al-Khalifa, et al., 2012) bringing into consideration a welfare factor. If the poultry industry is going to utilize this approach to better serve consumers, it is important that the birds' health is not compromised. Similarly, while there may be an advantage in enhancing the fatty acid profile of the meat, care must be taken to ensure that the sensory quality does not deteriorate (López-Ferrer, et al., 2001). According to González-Esquerra, et al., (2001) sensory quality of eggs and meat is decreased due to off flavors

and reduced lipid stability, which influences customer perception. Hence, the purpose of this review is to highlight the effect of utilizing n-3 fatty acids on bird performance, meat and egg quality, fat deposition and consumer preferences.

**Dietary fat types included in poultry diets**

In considering fat sources in broiler diets, cost and quality of the respective fats and impact on both animal performance and carcass quality have to be taken into account (Zollitsch, et al., 1997). Due to the fact that animal-based fat such as tallow and grease were readily available and inexpensive, they became the primary source of fat utilized often at very high levels (Siedler, et al., 1955). Despite their benefits as an energy source, animal-based fats are high in saturated fatty acids which have been shown to have a negative impact on human health (Woods, et al., 2009). This criticism resulted in the increased use of less saturated fat sources. Currently, vegetable oil such as sunflower oil, canola oil, soybean oil, and corn oil are the most common fat sources used in the poultry diet (Allahyari-Bake, et al., 2017). The increased use of more plant based fat was a welcomed change as consumers were recognizing that the type of fat they consume was affecting their health. Though adjustments have been made in the type of fat utilized, the fatty acid content of the poultry diet is still much greater in saturated fats and unsaturated fatty acids of the n- 6 fatty acids class compared to n-3 fatty acids.

**Impact of dietary fat type on performance and bird health**

If changes are made to traditionally-fed poultry diets, the impact on growth and performance must be a focus since any reduction in productivity would be costly to producers (López-Ferrer, et al., 2001). Since differences in digestibility affect animal performance (Zollitsch, et al., 1997), it is imperative to optimize fat source and intake with poultry performance to meet consumer demands. Results from studies feeding titrated levels of long chain polyunsaturated fatty

acids are conflicting hence no consensus has been reached on suitable inclusion rates. Overall, regardless of the fat source, bird performance and health are not negatively affected (Sanz, et al., 2000). In fact, lowering the levels of saturated and n-6 fatty acids in the diet may be more beneficial to the birds. Cortinas, et al., (2004) reported that performance of birds fed dietary polyunsaturated fatty acids was not altered; however, percentage thigh weight tended to increase as the level of unsaturation increased in the diet. Interestingly, Rahimi, et al., (2011) reported that feeding diets high in n-3 (flax seed and canola seed) had a negative effect on performance with birds having decreased body weight gain and greater feed conversion ratio despite having similar feed consumption. This difference however could be due to the fact that there may have been some anti-nutritional factors present in these oils as a result of the extraction process.

One potential area that could benefit greatly from feeding diets high in n-3 fatty acids is abdominal fat deposition. One negative result of the selection for fast growing broilers has been an increase in depositing carcass fat. Most of the excess fat is not physiologically essential and is discarded during slaughtering and processing or before cooking (Chambers, et al., 1981). In order for this fat to be economically beneficial, the fat must be removed and reprocessed as a component of poultry by-product meal at a low cost (Hood, 1982). Therefore, identifying a way to reduce excess fat deposition would be beneficial to the broiler industry. Studies have shown that birds fed n-3 fatty acids have reduced subcutaneous and abdominal fat. Crespo, et al., (2002) reported that diets high in polyunsaturated fatty acids reduced fat content of abdominal and subcutaneous fat which accounts for approximately 30% of the total body fat. Similarly Sanz, et al., (2000) reported that feeding a saturated fat source then substituting it with an unsaturated source for the last eight days may reduce fat deposition and improve fat fluidity compared to using a saturated source throughout their entire life. Though adiposity was not decreased, Torchon, et al., (2017) found that

birds fed a diet containing fish oil from 7 to 30 days had significantly smaller adipocytes compared to those fed diets containing oils high in n-6 fatty acids.

Earlier studies focused on the mechanisms that make n-6 fatty acids to be more pro-inflammatory compared to the n-3. However, recent studies have also evaluated the effects of fatty acids on gene expression (Simopoulos, 2000) and their influence in regulating fat deposition. Utilizing molecular techniques and computer software, researchers have been able to better identify mechanisms that play critical roles in fat deposition. Ailhaud, et al., (2004) reported that key transcriptional regulators of various lipid related genes are affected by n-3 fatty acids. One potential benefit has been the ability of n-3 fatty acids found in fish oil in lowering serum triacylglycerol levels hence reducing fat accumulation in the adipocyte (Guo, et al., 2005).

**Influence of dietary fat type on fatty acid profile and quality of meat and egg**

Diet manipulation by incorporating different oils or oil seeds in poultry diet is the usual way of increasing the n-3 fatty acid content of poultry products. Currently, the egg industry has made more progress in producing and marketing enriched products (González-Esquerra, et al., 2001). Omega-3 sources include plant and animal-based with the plant-based sources providing alpha linolenic acid while the marine sources provide eicosapentaenoic acid (EPA 20:5), docosapentaenoic acid (DPA, 22:5), and docosahexaenoic acid (DHA, 22:6) (Cherian, 2008a). According to López-Ferrer, et al., (2001) meat enriched in n-3 fatty acids, using all vegetable fat seems to be less effective compared to marine fat. Flax seeds or fish oil is commonly used to manipulate the n-3 fatty acid content of poultry foods. However, the efficiency of flax in increasing the content of long chain n-3 is limited after 10% inclusion in the bird's diet (Cherian, 2008b). Adding flax results in an increase in α-linolenic acid (18:3) in triglycerides and long chain n-3 fatty acids in the phospholipids (Cherian, 2008b). Similarly (Zuidhof, et al., 2009) found that

increasing the level or duration of feeding flaxseed decreased feed intake, body weight, and the carcass and breast yield. In a study comparing the feeding fish oil and sunflower oil (Newman, et al., 2002) found that, that birds fed fish oil had higher levels on n-3 in their phospholipid profile. On the other hand, birds fed sunflower oil, had higher levels of n-6 and had a fatty acid profile similar to birds fed tallow. Cortinas, et al., (2004) found that higher levels of polyunsaturated fatty acids in the diet resulted in higher accumulation in the thigh and breast. By feeding diets that varied in the level of polyunsaturated fatty acids, they were able to achieve EPA (2.05%) and DHA (0.99%) and EPA (3.17%) and DHA (2.79%) enrichment in the thigh and breast respectively.

**Influence of dietary fat source on consumer perception and health**

Long chain fatty acids, whether in adipose tissue or muscle, contribute to important aspects of meat quality and are central to the nutritional and sensory values of meat (Webb, et al., 2008). (Yang, et al., 2010) reported that fish oil tended to reduce ultimate pH of breast muscle. These authors also concluded that fish oil is liable to oxidization due to it being a rich source of long-chain PUFA. Similarly, Betti, et al., (2009) reported that susceptibility to oxidation increased in both breast and thigh broiler meat with the duration of feeding flaxseed. In addition, these authors reported that enriching the diet for less than 16 d did not result in perceivable sensory defects. However, it was concluded that the duration of flaxseed feeding significantly affected the color characteristics, functional properties, and oxidative stability of broiler meat.

## Summary and conclusion

Feeding diets high in n-3 polyunsaturated fatty acids to poultry to enrich meat and eggs could potentially be used as a means of improving the levels of n-3 fatty acids in the human diet. However if this method of enrichment is to be utilized, there are a number of factors that needs to be addressed. These include, identifying a suitable ingredient. The major source of long chain n-3 polyunsaturated fatty acids is fish oil. However, feeding this to poultry may be very costly. Hence

identifying an ingredient that has a similar of better fatty acid profile would be critical. The suitable ingredient must also generate the same or better quality poultry products therefore it is important that all areas of production be taken into consideration. Similarly, consumer preference and willingness to purchase is essential. Also, factors that affect n-3 deposition before and after processing and during cooking must be identified.

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# CHAPTER III.

# ENRICHING THE HEN DIET IN LONG CHAIN N-3 POLYUNSATURATED FATTY ACIDS PROGRAMS CHICKS TOWARDS REDUCED ADIPOSITY

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R.C.B performed the experiments, analyzed data and drafted the manuscript. S.J.H and S.D provided technical assistance. A.T.F and S.R.C performed Lipidomics analyses, and J.Y and R.H performed proteomics analyses. J.W provided access to hens and chicks. B.H.V directed the project, obtained funding, and reviewed and edited the manuscript and is the corresponding author.

## Abstract

Maternal intake of long chain n-3 polyunsaturated fatty acid (LC n-3 PUFA), particularly eicosapentaenoic acid (EPA; 20:5 n-3) and docosahexaenoic acid (DHA; 22:6 n-3) has been associated with reduced adiposity in offspring. We determined if enriching the developing embryo in LC n-3 PUFA influences adipose tissue development and metabolism. Chickens were used as a model because fatty acid content of the yolk, which supplies lipids to the developing embryo, can be manipulated through the hen diet. Cobb 500 hens (n=30/group) were fed broiler-breeder diets with fat (2.3 % fat wt:wt) from corn oil (CO; n-6) or fish oil (FO; n-3). After feeding hens for 28 d, fertilized eggs were collected and hatched. All chicks were fed a CO-based (3 % fat wt:wt) broiler starter diet (n=28/group). Body and adipose weights (abdominal and subcutaneous) were measured at 7 and 14 d of age. Abdominal adipose tissue was used to measure adipocyte size, gene expression (QPCR), fatty acid composition (GC-MS), and proteomics (LC-MS/MS). Data were analyzed by ANOVA or t-test. Maternal FO significantly enriched chick adipose tissue in EPA and DHA and reduced adiposity (fat pad wt/body wt). At 14 d adiposity was reduced in abdominal

and subcutaneous depots by 38% and 40%, respectively in FO vs CO chicks ($P<0.05$). Adipose tissue of FO chicks was characterized by reduced adipocyte size, upregulated peroxisome proliferator activated receptor gamma (PPAR-γ) and PPAR-γ coactivator 1 beta (PPARGC1B) expression (each ~ 4-fold; $P<0.001$) and reduced expression of lipoprotein lipase (LPL) compared to CO chicks. Proteomics identified 95 differentially abundant proteins, including components of glucose metabolism and cytoskeletal organization. These results demonstrate that enriching tissues of the developing chick embryo in LC n-3 PUFA influences adipose development and reduces fat mass after birth.

Keywords: Obesity, n-3 Polyunsaturated Fatty Acid, developmental programming, adipose tissue

## Introduction

Active proliferation and differentiation of preadipocytes shortly before birth and in the first few years of life creates a sensitive window for adipose development (Baum, et al., 1986; Knittle, et al., 1979; Salans, et al., 1973). Consequently, the maternal diet and in utero environment can impact adipose deposition and the consequent risk for obesity. Adipose tissue is subject to developmental programming, in which dietary and environmental factors in utero exert lasting effects on tissue phenotypes. Variations in the maternal diet, lifestyle and environmental exposures have been linked to increased adiposity later in life through stable effects on adipocyte growth and metabolism (Bruce, et al., 2010; Lisboa, et al., 2012; Lukaszewski, et al., 2013). Programming of adipose tissue is of particular public health interest because obesity, which is epidemic in the U.S. and globally, begins early in life. Approximately 27% of children in the U.S. are overweight or obese by age five (Cunningham, et al., 2014). Obese children are much more likely to be obese as adults when compared to normal weight children (Freedman, et al., 2001; Guo, et al., 1999). Therefore, limiting excess fat accumulation in the first few years of life is an important tool for the

prevention of adult obesity. Evidence that adiposity at birth predicts fatness later in childhood highlights the need to understand prenatal factors that influence adipose development (Catalano, et al., 2009; Wang, et al., 2016).

The types of fatty acids provided in the maternal diet may influence early adipose development and the resultant propensity for fat accumulation in children. In vitro studies with adipocyte cell lines demonstrate that polyunsaturated fatty acids (PUFAs) of the n-3 and n-6 series differentially regulate preadipocyte proliferation, adipogenesis, and triglyceride storage. Omega-6 PUFAs, particularly arachidonic acid (AA; 20:4 n-6) tend to be pro-adipogenic (Gaillard, et al., 1989; Massiera, et al., 2003; Negrel, et al., 1989), while LC n-3 PUFAs (e.g., eicosapentaenoic acid (EPA; 20:5 n-3) and docosahexaenoic acid (DHA; 22:6 n-3) attenuate lipid accumulation and promote an oxidative adipocyte phenotype (Fleckenstein-Elsen, et al., 2016; Kim, et al., 2006; Manickam, et al., 2010). Fatty acid profiles of diets in the US and other industrialized countries have shifted over the last several decades to favor consumption of n-6 PUFAs at the expense of n-3 PUFAs (Blasbalg, et al., 2011). Both fatty acids supplied to the developing embryo and the fatty acid profiles of breast milk directly reflect the maternal diet (Arterburn, et al., 2006), creating the potential to impact the earliest stages of adipose development. Epidemiological attempts to associate maternal dietary fatty acid profiles with fat mass in children have been inconclusive. Two recent prospective studies demonstrated an inverse relationship between levels of n-3 PUFAs in maternal blood during pregnancy and fatness in childhood (Donahue, et al., 2011b; Vidakovic, et al., 2016). However, relative contributions of the pre- and perinatal maternal diet are difficult to separate from shared consumption patterns after lactation in human studies.

Avians provide a unique model in which to specifically manipulate the pool of fatty acids that are supplied to the embryo and test the effects on adipose deposition after hatch (i.e. birth).

The yolk provides the majority of fatty acids to developing tissues in the embryo, and for one to two days after hatch, until feeding is established. The fatty acid profile of the yolk can be modified through the source of dietary fat provided to the hen (Cherian, et al., 1996; Hargis, et al., 1991). For example, commercial eggs that are enriched in EPA and DHA are produced by supplementing the hen's diet with marine oils. We used this relationship to test the hypothesis that enriching the embryo in EPA and DHA, supplied in fish oil, reduces adipose deposition in chicks. Corn oil was used as a reference because it contains a comparable level of PUFA (~ 60%), but primarily those of the n-6 family. All chicks were fed a corn oil-based diet after hatch to confine the experimental manipulation to the period of embryonic development. We demonstrate that maternal fish oil feeding significantly reduced adiposity after hatch, with no effect on growth. Our results suggest that fatty acids in the maternal diet contribute to developmental programming of adipose tissue.

## Materials and Methods

### Diets and husbandry

Animal husbandry procedures were reviewed and approved by the Institutional Animal Care and Use Committees of the University of Georgia (broiler breeder hens) and the University of Tennessee (chicks). Cobb 500 broiler breeder hens maintained at the University of Georgia poultry unit were fed commercially-formulated broiler breeder diets in which fat (2% wt:wt) was provided from either fish oil (Menhaden) (FO) (Icelandirect; Clifton, NJ) or corn oil (Wesson), (CO) (Conagra Brands; Chicago IL) (n = 30 hens/diet). Diet composition is shown in Table 3.1. After 28 d, fertilized eggs were collected from each hen and transported to the University of Tennessee for incubation and hatching. Multiple roosters were used for fertilization of eggs in each diet group. Eggs were weighed and incubated for three weeks, until hatch. Hatch rates were calculated for each group as a percentage of eggs that produced viable chicks. At hatch, chicks were grouped by hen diet (CO or FO) and housed separately in brooder cages (n=10/cage) at

standard brooding temperature 35$^o$ C. Each cage was equipped with a feeder and drinker to which birds had ad libitum access. Both CO and FO chicks were fed a standard broiler starter diet in which fat (3% fat wt:wt) was supplied from CO. Weight gain and feed intake were monitored weekly.

**Blood and tissue collection**

Chicks were euthanized by $CO_2$ asphyxiation. Two chicks from each group were euthanized at hatch for collection of liver and brain for lipid analyses. Samples of each tissue were snap-frozen and stored at -80$^o$ C. The remaining chicks were euthanized at 7 and 14 d of age. At the time of euthanasia blood was collected by cardiac venipuncture and transferred to 10 ml serum separator tubes (Fisher Scientific, Pittsburgh, PA). Serum was separated by centrifugation and stored at -80$^o$ C until analyses of circulating metabolites. Abdominal and femoral (subcutaneous) adipose depots were dissected and weighed as indices of adiposity. Samples of each depot and of liver were subsequently snap-frozen in liquid nitrogen and stored at -80$^o$C. Samples of abdominal adipose tissue were fixed for 24 h at 4$^o$C in paraformaldehyde (4%) for determination of adipocyte size by histology.

**Serum metabolites**

Commercially available colorimetric assay kits were used to measure serum glucose (Cayman Chemical, Ann Arbor, MI) and non-esterified fatty acid (NEFA) levels (Wako Chemicals, Neuss, Germany).

**Fatty acid analysis**

Abdominal fat samples from five randomly selected birds in each diet and age were analyzed for fatty acid composition by GC. Tissues (approximately 50mg) were pulverized under liquid nitrogen using a stainless steel mortar and pestle. Analyses were performed by the W.M.

Keck Metabolomics Research Laboratory (Iowa State University, Des Moines, IA). Data for each fatty acid were expressed as mole% ±SEM.

**Phospholipid analysis**

Fatty acid composition of phosphatidylcholine species in brain and liver collected at hatch (n=2/diet) and in abdominal adipose tissue collected at 7 d of age (n=5/diet) was analyzed using UPLC-MS. Tissue samples (100mg) were pulverized under liquid nitrogen using a mortar and pestle. Phospholipids were extracted using a modified Bligh and Dyer protocol (Milne, et al., 2006). Dried extracts were resuspended in 300µL of methanol/chloroform (9:1) for UPLC-MS analysis as described by (Campagna 2017).

Lipids were identified with high resolution mass spectrometry using exact *m/z* and retention times. Lipid standards (Avanti Polar Lipids, Alabaster AL) from each phospholipid class were run to verify retention times. All ion fragmentation was used to confirm that phosphatidylcholines contained DHA and EPA as acyl chains. For all ion fragmentation scans, the resolution was 140,000 with a scan range of 100-1500 *m/z*. The normalized collision energy was 30eV with a stepped collision energy of 50%. Lipids were identified by their fragments using Xcalibur software (Thermo Fisher Scientific, San Jose, CA). Data analysis was performed using Maven software (Clasquin, et al., 2012).

**Adipocyte size**

Abdominal fat samples from three birds/diet at each of the two ages were embedded, sectioned and stained with hematoxylin and eosin (two slides/bird) for determination of adipocyte size, as previously described by (Ji, et al., 2014). Briefly, images of three independent fields were captured on each slide under 20x magnification with the Advanced Microscopy Group EVOS XL Core microscope (Fisher Scientific, Pittsburgh, PA). For consistency, the same person performed

measurements. Image J (Version 1.48, National Institutes of Health) was used to determine adipocyte area, ($\mu m^2$), using microscope settings of 2.8 μm/pixel, and using the restriction that measurements must exceed 500 $\mu m^2$. Adipocytes were grouped into one of six arbitrary bins and the frequency of cells within each bin was determined. A standard method was used to calculate adipocyte number from adipocyte volume and adipose mass (Di Girolamo, et al., 1971).

**Real time PCR assay**

Total RNA was isolated from approximately 200 mg of abdominal adipose tissue and liver from five chicks in each treatment using Invitrogen™ TRIzol™ (Invitrogen, Carlsbad, CA). CDNA was synthesized from 500 ng total RNA in 20 µl reactions using iScript cDNA Synthesis kit (Bio-Rad Laboratories, Hercules, CA). Predesigned and validated primers for quantitative real-time PCR (QPCR) were purchased from Qiagen (Quantitect; Germantown, MD). QPCR was performed in triplicate for each sample using iQ SYBR Green Master Mix (Bio-Rad Laboratories, Hercules, CA), as previously described (Ji, et al., 2014). Expression levels of genes of interest were normalized to expression of TBC1 domain family 8 used as a housekeeper.

**Proteomics**

Approximately one g of abdominal adipose tissue from each of three chicks per diet was pulverized in liquid nitrogen, of which approximately 60 mg was used for protein extraction. Proteins were extracted using a detergent-free, methanol/chloroform (2:1) protein extraction protocol (Vaisar, 2009) designed for lipid-rich tissues and based on the Bligh and Dyer method (Bligh, et al., 1959). Proteins were precipitated from the aqueous fraction using trichloroacetic acid and digested with sequencing grade trypsin. Approximately 2 mg of proteolytic peptides were obtained from each sample after clean up. Fifty µg aliquots of these peptides were used for 2D-LC-MS/MS proteomic measurements on an LTQ Orbitrap mass spectrometer (Thermo Fisher), as

previously described (Li, et al., 2014). MyriMatch v2.1.111 (Tabb, et al., 2007) was used to search the raw mass spectra against the predicted protein database to identify fully-tryptic peptides, which were then grouped together into respective proteins with IDPicker v.3 (Ma, et al., 2009). Only protein identifications with at least two identified peptide spectra and a maximum q-value of 0.02 were considered for further analysis. Peptide fragments were mapped to proteins in the *Gallus gallus* genome (V3.0) using Uniprot.

**Statistical Analysis**

The experimental design was a completely randomized design split plot with diets in the whole plots and age (d) in the subplots. Data collected at both 7 and 14 d (Weights, serum metabolites, tissue fatty acid composition, average adipocyte size and number) were analyzed using mixed model ANOVA with terms for diet, age and their interaction. Significant F-test ($P < 0.05$) was followed by post-hoc testing using least square means to identify pairwise differences between groups. Adipocyte size frequency distributions within each age were further compared using t-test ($P < 0.05$) as were data collected at one age (QPCR, proteomics). Identified protein spectra counts were normalized and analyzed in MetaboAnalyst 3.0 (Xia, et al., 2015). Functional enrichment analysis was performed using the Database for Annotation, Visualization and Integrated Discovery (DAVID, V 6.8) (Huang, et al., 2008).

## Results

**Egg production and chick fatty acid composition**

Hatchability, egg weights, and chick weight at hatch were used to assess the effect of hen diet on egg quality, none of which differed significantly between eggs from CO and FO hens ($P>0.05$). The fatty acid composition of phospholipids contained in brain and liver was profiled to confirm that EPA and DHA were enriched in tissues of FO chicks, compared to CO chicks, at hatch. Brain and liver were used because of their relative mass at hatch. Enrichment levels of

phosphatidylcholine (PC) species containing EPA and DHA are shown in Table 3.2. The fold-increase (FO/CO) ranged from ~ 1.2 (18:0/22:6 in brain) to ~ 130.4 (PC 18:4/22:6 in liver), with greater than 2-fold enrichment for most species. These data confirm that the maternal dietary fatty acid profile influenced was reflected in tissue fatty acid composition of the resultant chicks at the time of hatch.

Gas chromatography-mass spectrometry (GC-MS) was used to quantify the effect of maternal fatty acid source on the fatty acid composition of developing adipose tissue. Fatty acid composition of the total lipid fraction of abdominal adipose tissue was analyzed at 7 and 14 d of age (Table 3.3). Tissue abundance of five fatty acids (palmitoleic, γ-linolenic, eicosenoic, eicosadienoic and docosanoic acids) increased with age ($P<0.05$) but was not affected by diet or age*diet interactions. Adipose tissue of FO chicks was significantly enriched in EPA and DHA at both ages ($p_{Diet} <0.01$). At 7 d, tissue from FO chicks contained approximately six times more EPA than tissue from CO chicks. Enrichment declined from 7 to 14 d as all chicks consumed a corn oil-based diet ($p_{Age*Diet} < 0.01$), but still differed by ~ 2-fold. Comparable effects were seen for DHA content, which was increased by ~ 2.5-fold at 7 d and ~ 1.8-fold at 14 d ($p_{Diet} <0.01$). As for EPA, the relative enrichment declined with age ($p_{Age*Diet} =0.03$). Fatty acid composition of PC species in abdominal adipose tissue at 7d also reflected the maternal diet. Five species containing EPA or DHA were significantly increased, by 3- to 4-fold, in FO vs. CO tissue (Table 3.3).

**Body weight and adipose deposition**

Subcutaneous adipose tissue in chickens develops in the embryo and is visible at hatch, while the abdominal depot develops in the first few days after hatch. Weights of both depots were measured to assess the effect of FO in the maternal diet on offspring fat deposition. Body weights did not differ significantly between CO and FO chicks at 7d, but FO chicks were significantly

heavier than CO chicks at 14 d (Table 3.4). Maternal fatty acid source regulated adiposity of both depots in an age-specific manner. At 7 d, subcutaneous and abdominal adiposity did not differ significantly between FO and CO chicks ($P>0.05$). However, relative weights of both depots were significantly reduced in FO vs. CO chicks ($P<0.05$) at 14 d. On average, FO reduced adiposity by ~ 38% in each of the two depots. Effects of maternal diet on adiposity were not associated with diet-induced differences in glycemia or lipolysis, as plasma glucose and NEFA levels were comparable between treatments at both ages (Table 3.4). The effects of maternal FO on abdominal adipose tissue were further explored because this is the depot in which broilers primarily deposit excess fat. Adipocyte size in H&E-stained sections of abdominal adipose tissue was measured to determine if fatness between groups differed due to hypertrophy. As expected, adipocyte size increased from 7 to 14 d, reflecting the rapid increase in fat deposition (Fig. 3.1). Average adipocyte size did not differ between FO and CO at 7 d ($P = 0.21$) but was significantly reduced in FO at 14 d ($P = 0.03$) Adipocyte number, calculated based on adipocyte size and adipose mass, showed a corresponding significant increase in FO vs. CO chicks at 14 ($P=0.03$) but not 7 d of age ($P=0.12$). Analysis of the adipocyte size distribution revealed that FO favored the abundance of small adipocytes, while there was a greater frequency of larger adipocytes in the CO chicks (Fig. 3.1). At 7 d there was a significant increase in the percentage of adipocytes in the three larger bin sizes in the CO chicks ($P < 0.05$; Fig.3.1A). This effect persisted at 14 d with approximately a 3-fold increase in the percentage of adipocytes ($P = 0.02$) in the largest bin (Fig. 3.1B). Conversely, approximately 66% of FO adipocytes were in the two smallest bins, compared to 49% in CO.

**Quantitative Polymerase Chain Reaction**

Potential mechanisms for the difference in adiposity were evaluated based on expression of genes that mediate fatty acid metabolism and adipogenesis. As shown in Fig. 3.3 A, adipose

tissue from FO chicks expressed significantly higher levels of peroxisome proliferator activated receptor-γ (PPARγ; 4.4-fold) and its coactivator PPARGC1B (PPARγ coactivator 1 β; 3.4-fold) than tissue from CO chicks ($P<0.05$). Conversely, expression of lipoprotein lipase (LPL) was approximately 60% lower in adipose tissue from FO vs. CO ($P<0.05$). Expression of carnitine palmitoyltransferase 1 (CPT1; mitochondrial fatty acid oxidation), acyl-coenzyme A oxidase 1 (ACOX1; peroxisomal fatty acid oxidation), and fatty acid synthase (FASN; de novo lipogenesis) did not differ significantly between groups (Figure. 3.3 B; $P>0.05$). Liver is the primary site of de novo lipogenesis in avians (as in humans) (Leveille, et al., 1968) and plays an important role in fat deposition in broiler chickens (Leclercq, 1984). Diet did not significantly affect expression of CPT1, ACOX1 and FASN in liver (Figure 3.3 C), consistent with comparable hepatic triglyceride content in FO and CO chicks (data not shown).

**Adipose tissue proteomics**

The proteomes of adipose tissue from FO and CO chicks were compared to identify additional pathways that were altered by maternal FO feeding. A total of 95 known proteins differed significantly ($P<0.05$) between FO and CO chicks (Table 3.5). Functional enrichment analysis revealed that this set of proteins was enriched (adj. p-value $<0.05$) for components of cytoskeletal organization and cellular tight junctions, and for functions relevant to glycolysis and gluconeogenesis (Table 3.5). Proteins associated with the cytoskeleton (e.g., actin, vimentin, tubulin) were more abundant in FO vs. CO adipose tissue. Maternal FO also increased levels of the adipocyte lipid droplet protein perilipin (PLIN1) and Ras-related protein Rab-18 (RAB18; both ~ 1.9-fold, FO/CO), although these changes did not meet the criterion for statistical significance ($p=0.051$ and 0.053, respectively). Glycolytic proteins were downregulated by maternal FO. Fructose-1, 6-bisphosphatase 1 and 2 (FBP1 and FBP2), pyruvate kinase (PK), enolase 3 (ENO3),

and phosphoenolpyruvate carboxykinase 2 (PCK2) were significantly less abundant in tissue of FO vs. CO chicks. Two fatty acid binding proteins, fatty acid binding protein1 (FABP1) and liver basic fatty acid binding protein (LBFABP), were also present at significantly lower levels in FO adipose tissue. Functional enrichment indicated that proteins involved in muscle development and filament structure differed between FO and CO chicks.

## Discussion

This study fills a gap in knowledge by demonstrating that the types of fatty acids supplied to the developing embryo before birth influence adipose development. More specifically, enriching the maternal diet in FO reduced chick adiposity when compared to a maternal diet based on CO. All chicks were fed a CO-based diet after hatch, restricting the dietary manipulation to the period prior to hatch The feeding protocol that we used was developed to enrich eggs for the consumer market in DHA and EPA. Although we did not measure yolk fatty acids, we did find marked enrichment of EPA and DHA in liver and brain phospholipids at hatch, indicating diet enriched the developing embryo as expected.

Attempts to retrospectively link fatty acid content of the maternal diet to child adiposity in humans have been inconclusive (Muhlhausler, et al., 2010). These types of studies are limited by reliance on BMI, which is a coarse index of adiposity in children, and on dietary recall to assess fatty acid intake. However, recent prospective studies using more sensitive measures of body composition and of fatty acid status have demonstrated an inverse relationship between maternal AA/DHA+EPA levels and childhood adiposity. A study of 227 mother-child pairs revealed that the ratio of AA/DHA+EPA in maternal circulation mid-pregnancy predicted adiposity in children at three years of age (Donahue, et al., 2011a). In a much larger study (4,830 mother-child pairs), Vidakovic, et al., (2016) reported that mid-pregnancy levels of EPA, DHA and docosapentaenoic acid (DPA) (22:5 n-3) pregnancy were associated with lower percentage of body fat and abdominal

fat in children at a median age of six years. Likewise, maternal levels of n-6 PUFA were associated with increased childhood adiposity. The ratio of AA/DHA+EPA in transitional breast milk, which reflect dietary patterns in the previous 90 days, was also recently shown to predict body fat percentage at four months of age (Rudolph, et al., 2016). Interestingly, none of these studies found a significant relationship with body weight or BMI, just as we found no effect of maternal FO feeding on chick body weight or growth, suggesting specific effects on adipose tissue. Each of these studies profiled fatty acids in the maternal blood, which provides a sensitive assessment of intake during pregnancy. However those levels may not completely reflect fatty acid delivery to the embryo, which also depends upon transfer across the placenta (Hornstra, 2000). They also may be confounded by the child's dietary intake after birth and lactation, particularly those that measured adiposity a few years after birth. Our findings complement these studies by demonstrating that fatty acids provided during embryonic development alone are sufficient to alter adiposity.

The shift towards increased small adipocytes coupled with upregulation of PPAR-γ and its coactivator PGC-1β suggest that maternal FO promoted adipogenesis, despite reducing adiposity. EPA and DHA can act as ligands to activate PPAR-γ, which would be expected to promote adipogenesis through this nuclear receptor's role in orchestrating adipocyte differentiation. However, in vitro studies have shown both pro-and anti-adipogenic effects of EPA and DHA, which may be due to variation in cell lines, differentiation protocols, reference treatments, and fatty acid concentrations (Chambrier, et al., 2002; Murali, et al., 2014; Oster, et al., 2010). Interestingly, a shift towards increased frequency of small adipocytes and increased expression of PPAR-γ has been described in fat-1 mice, which endogenously synthesize n-3 PUFA due to transgenic expression of a novel fatty acid desaturase from C. elegans (White, et al., 2015).

Microarray data indicated that constitutive synthesis of n-3 PUFAs within adipocytes of fat-1 mice markedly suppressed expression of GATA3, which normally inhibits the progression of preadipocytes into differentiation by directly suppressing PPAR-γ (Tong, et al., 2000).

Reduced availability of glycerol-3-phosphate and fatty acids for triacylglycerol synthesis may have restricted hypertrophy of FO adipocytes. Esterification of fatty acids into triacylglycerol requires a steady supply of glycerol-3-phosphate. In adipose tissue, this is synthesized during glycolytic metabolism of glucose, and from pyruvate through glyceroneogenesis (Chaves, et al., 2006; Forest, et al., 2003). Levels of several glycolytic proteins and of PEPCK, which is rate-limiting for glyceroneogenesis, were reduced in FO vs. CO adipose tissue. In chickens (and humans) the majority of stored fatty acids are delivered to adipose tissue from the liver. Lipoprotein lipase, which cleaves fatty acids from circulating lipoproteins, was also down-regulated in FO adipose tissue. In combination, these effects suggest that maternal FO may have reduced adipocyte size, at least in part, by attenuating the capacity to extract, esterify and store fatty acids as triacylglycerol. Whether this is a primary effect of maternal FO or a secondary response to reduced delivery of fatty acids from liver cannot be determined, as we did not measure plasma VLDL levels. However, hepatic lipogenesis (based on expression of FASN) and triglyceride content (data not shown) did not differ between FO and CO, suggesting that diet did not alter the supply of fatty acids from liver.

A growing body of literature illustrates that the structural assembly of lipid droplets influences lipid metabolism in adipocytes. Maternal FO feeding increased the abundances of three proteins, PLIN1, VIM and RAB18, which localize to the surface of adipocyte lipid droplets and play key roles in balancing lipid storage and mobilization. Perilipin1 is the major surface protein of adipocyte lipid droplets, where it orchestrates lipolysis by controlling access of lipase enzymes

to triacylglycerol molecules (Blanchette-Mackie, et al., 1995a; Blanchette-Mackie, et al., 1995b; Londos, et al., 1996). Vimentin is an intermediate filament that scaffolds lipid droplets to maintain their individual structural integrity (Schweitzer, et al., 1998). Rab18 facilitates exchange of lipids between lipid droplets and the endoplasmic reticulum, playing roles in both lipolysis and lipogenesis (Martin, et al., 2005; Ozeki, et al., 2005). All three proteins are critical for the physical remodeling and trafficking that are necessary for storage and mobilization of lipids. The physiological significance of increased levels of vimentin, perilipin1 and Rab18 in FO adipose tissue requires further study, but the roles of each protein and their interactions in lipid mobilization suggests that this response may facilitate lipid utilization within adipocytes. Consistent with this possibility, overexpression of perilipin was shown to induce fatty acid oxidation in white adipocytes (Sawada, et al., 2010). Follow-on studies are prompted to determine how maternal FO influences adipocyte architecture and if this contributes to the reduction in adiposity.

The mechanisms through which maternal consumption of LC n-3 PUFA can reduce adiposity in offspring remain to be determined. Transcriptional control through PPARs by EPA, DHA and their metabolites would require sustained enrichment of those fatty acids within adipose tissue. Fatty acid profiling demonstrated that the total lipid pool of FO chicks was enriched in both EPA and DHA up to 14 d, although the fold-enrichment relative to the CO group decreased between weeks one and two. How long this enrichment persists, especially when the post-hatch diet is not supplemented with FO, remains to be determined. Epigenetic modifications of genes involved in adipose deposition may also underlie reduced adiposity in FO chicks. In a recent randomized, controlled clinical study, fish oil supplementation during pregnancy was shown to differentially methylate 21 chromosomal regions at birth, with some differences persisting to five years of age (van Dijk, et al., 2016). Circulating DHA levels, both early in pregnancy and at birth,

were significantly correlated with methylation of PPAR-α in infants (Marchlewicz, et al., 2016), indicating the potential for epigenetic programming of lipid metabolism. Follow-on studies to characterize methylation patterns and other epigenomic marks of maternal FO feeding are needed to explore this possibility.

Chickens provide a unique means to address maternal programming by dietary fatty acids, but several caveats and limitations should be noted. Fish oil and CO were the sole sources of dietary fatty acids in our model. This was intentional, to maximize enrichment of the yolk in EPA and DHA. However, fatty acid consumption in human diets is more diverse and complex, and the level of enrichment we achieved may not occur in the context of a typical human diet. Nevertheless, our results provide proof-of-principle that fatty acids provided prior to birth regulate adipose development. As such, they contribute additional rationale for further studies in humans to determine if maternal fatty acid intake can be used to reduce the risk of childhood obesity. Intake of most fish species during pregnancy is now encouraged by the US Food and Drug Administration, easing previous concerns about safety of fish oil intake through foods (2017). Our study was also limited to measuring adiposity within a short period (up to 14 days) after hatch, so the longevity of reduced fatness is unknown. Recent studies linking maternal levels of EPA and DHA during pregnancy with child fatness at up to six years of age encourage the interpretation that the programming effects of FO may persist. It should also be noted that chicks in the FO group were significantly heavier at 14 (but not 7) days of age, despite having significantly less adipose tissue. We infer that this difference in weight was due to differences in breast muscle, which grows at its fastest rate in this age span and accounts for 10-15% of body weight (Scheuermann, et al., 2003). However we did not weigh muscle in this study. Further studies are necessary to evaluate

differences in lean growth in our model, and the potential effects of maternal FO on development of other tissues.

In summary, our data demonstrate that maternal fish oil consumption reduces adipose deposition in offspring. Our study was limited to the first two weeks of life, and follow-on experiments are necessary to determine how long this effect persists as chicks mature. These results complement recent studies in humans that link LC n-3 PUFA in the maternal diet to reduced adipose mass in children. Accordingly, they highlight the potential to attenuate fat accumulation and potentially the risk for childhood obesity through dietary intervention prior to birth.

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## Appendix

Table 3.1. Composition of FO and CO broiler breeder diets fed to hens for 28 d

| Ingredients | Amount, % | |
|---|---|---|
| | FO | CO |
| Corn | 63.16 | 63.16 |
| Soybean meal (48 %) | 11.85 | 11.85 |
| Wheat midds | 8.00 | 8.00 |
| Limestone | 7.90 | 7.90 |
| Pro-pak[1] | 5.00 | 5.00 |
| Fish oil | 2.30 | - |
| Corn Oil | - | 2.30 |
| Mono calcium phosphate | 0.60 | 0.60 |
| Termin-8[2] | 0.30 | 0.30 |
| Salt | 0.20 | 0.20 |
| Vit Pre-mix[3] | 0.20 | 0.20 |
| Bicarbonate of Soda | 0.15 | 0.15 |
| DL-Methionine | 0.14 | 0.14 |
| Choline | 0.12 | 0.12 |
| TM Pre-mix[4] | 0.08 | 0.08 |
| *Calculated nutrient analysis* | | |
| ME, kcal/kg | 2938 | 2938 |
| CP, % | 15.20 | 15.20 |
| Calcium, % | 3.5 | 3.5 |
| Total phosphorus | 0.61 | 0.61 |
| Available phosphorus | 0.43 | 0.43 |

[1]FO, fish oil; CO, corn oil

[1]Protein concentrate, H.J. Baker and Bros., 595 Summer Street, Stamford, CT 06901-1407 [2]Antimicrobial preservative

[3]Vitamin mix provided per kilogram of complete diet: vitamin A, 30,800 IU; Vitamin $D_3$, 9,250 IU; vitamin E, 153.9 IU; vitamin $B_{12}$, 0.154 mg; riboflavin, 46.2 mg; niacin, 185 mg; pantothenic acid, 84 mg; menadione sodium bisulfite, 16.2 mg; folic acid, 12.3 mg; pyridoxine HCl, 46.2 mg; thiamine HCl, 20.5 mg; biotin, 9.3 mg; choline, 2,944 mg; niacin, 185 mg

[4]Mineral mix provided per kilogram of complete diet: Cu, 55 mg; I, 7.3 mg; Fe, 366 mg; Mn, 310 mg; Zn, 321 mg; K, 2.23 g; Mg, 1.09 g; Se, 0.48 mg

Table 3.2. Enrichment of brain and liver phospholipids at hatch in broiler chicks produced from hens fed diets containing FO or CO for 28 d

| | FO/CO ratio | |
|---|---|---|
| PC species | Brain | Liver |
| PC(38:10) | 7.40 | 130 |
| PC(36:5) | 13.9 | 13.9 |
| PC(34:5) | 21.5 | 11.1 |
| PC(42:11) | 11.4 | 6.62 |
| PC(34:6) | 4.98 | 4.79 |
| PC(38:8) | 6.29 | 4.76 |
| PC(44:12) | 1.65 | 4.01 |
| PC(40:6) | 1.19 | 2.51 |
| PC(38:7) | 2.16 | 2.43 |
| PC(36:6) | 1.85 | 2.09 |
| PC(40:9) | 2.41 | 1.71 |

FO, fish oil; CO, corn oil N=2/group

Table 3.3. Abdominal adipose tissue fatty acid content of broiler chicks produced from hens fed diets containing FO or CO for 28 d

| Diet | CO | | FO | | | Effect | | |
|---|---|---|---|---|---|---|---|---|
| Age | 7 d | 14 d | 7 d | 14 d | SEM | Diet | Age | Diet x Age |
| Fatty Acid | % of total | | | | | P-value | | |
| Capric (C10:0) | 0.007 | 0.007 | 0.005 | 0.009 | 0.001 | 0.28 | 0.45 | 0.28 |
| Lauric (C12:0) | 0.024 | 0.020 | 0.024 | 0.027 | 0.002 | 0.13 | 0.81 | 0.13 |
| Myristic (C14:0) | 0.520 | 0.462 | 0.555 | 0.577 | 0.031 | 0.06 | 0.70 | 0.21 |
| Myristoleic (C14:1) | 0.144 | 0.154 | 0.152 | 0.195 | 0.015 | 0.13 | 0.11 | 0.31 |
| Pentadecanoic (C15:0) | 0.058 | 0.056 | 0.060 | 0.054 | 0.007 | 0.10 | 0.61 | 0.77 |
| Palmitic (16:0) | 22.43 | 22.13 | 23.42 | 28.86 | 1.920 | 0.61 | 0.20 | 0.15 |
| Palmitoleic (C16:1) | 5.170 | 6.090 | 5.610 | 7.94 | 0.610 | 0.08 | 0.01 | 0.27 |
| Heptadecanoic(C17:0) | 0.096 | 0.081 | 0.095 | 0.085 | 0.006 | 0.94 | 0.33 | 0.83 |
| Stearic (C18:0) | 5.723 | 5.032 | 5.430 | 6.381 | 0.509 | 0.49 | 0.87 | 0.29 |
| Oleic (C18:1) | 30.67 | 30.02 | 31.22 | 18.15 | 4.36 | 0.21 | 0.14 | 0.17 |
| Linoleic (C18:2n6c) | 16.45 | 17.16 | 15.93 | 17.979 | 1.378 | 0.99 | 0.39 | 0.55 |
| Linoleladic (18:2n6t) | 16.45 | 16.81 | 15.60 | 17.613 | 1.781 | 0.99 | 0.39 | 0.55 |
| G-Linolenic (18:3n6) | 0.180 | 0.206 | 0.164 | 0.209 | 0.015 | 0.68 | 0.03 | 0.55 |
| Alpha linolenic (18:3n3) | 0.718 | 0.672 | 0.689 | 0.722 | 0.067 | 0.88 | 0.92 | 0.56 |
| Arachidic (20:0) | 0.052 | 0.056 | 0.052 | 0.071 | 0.006 | 0.26 | 0.11 | 0.30 |
| Eicosenoic (C20:1n9) | 0.289 | 0.367 | 0.280 | 0.439 | 0.027 | 0.27 | <0.01 | 0.16 |
| Eicosadienoic (C20:2) | 0.134 | 0.155 | 0.137 | 0.169 | 0.010 | 0.45 | 0.03 | 0.60 |
| Eicosatrienoic (C20:3n3) | 0.131 | 0.142 | 0.143 | 0.166 | 0.001 | 0.12 | 0.15 | 0.60 |
| Eicostrienoate | 0.205 | 0.189 | 0.145 | 0.145 | 0.001 | 0.07 | 0.78 | 0.77 |
| Eicosapentaenoic (C20:5n3) | 0.009[b] | 0.010[b] | 0.060[a] | 0.02[a] | 0.005 | <0.01 | <0.01 | <0.01 |
| Erucic (C22:1n9) | 0.015 | 0.013 | 0.014 | 0.016 | 0.001 | 0.29 | 1.00 | 0.20 |
| Docosanoic (C22:2) | 0.031 | 0.037 | 0.031 | 0.043 | 0.003 | 0.29 | <0.01 | 0.36 |
| Tricosanoic (C23:0) | 0.050 | 0.035 | 0.030 | 0.029 | 0.006 | 0.045 | 0.20 | 0.25 |
| Docosahexaenoic acid (C22:6n3) | 0.036[b] | 0.016[b] | 0.092[a] | 0.028[b] | 0.007 | <0.01 | <0.01 | 0.03 |
| Nervonic (C24:1n9) | 0.017 | 0.006 | 0.008 | 0.008 | 0.004 | 0.39 | 0.17 | 0.20 |
| Activity index[1] | | | | | | | | |
| SCD-16 | 0.226 | 0.286 | 0.240 | 0.282 | 0.028 | 0.85 | 0.08 | 0.75 |
| SCD-18 | 5.412 | 7.474 | 5.902 | 3.680 | 1.316 | 0.23 | 0.95 | 0.12 |
| D6D | 0.006 | 0.01 | 0.004 | 0.010 | 0.002 | 0.57 | 0.01 | 0.57 |
| DNL | 0.683 | 0.646 | 0.753 | 0.084 | 0.132 | 0.11 | 0.91 | 0.50 |
| EL | 0.260 | 0.220 | 0.232 | 0.216 | 0.019 | 0.40 | 0.15 | 0.53 |

FO, fish oil; CO, corn oil; N= 5/diet/age; Effect (diet, age, and diet X age) P-value from ANOVA; Labeled means in a row without a common letter differ; $p<0.05$. [1]Indices of

enzyme activities calculated from mol%, using the following equations: SCD (Stearoyl-CoA desaturase)-16 (16:1n-7/16:0); SCD-18 (18:1n-9/18:0); D6D (Delta-6 desaturase) (18:3n-6/18:2n-6); DNL (De novo lipogenesis) (16:0/18:2n-6); EL (Elongation) (18:0/16)

Table 3.4. Effect of dietary enrichment on performance and serum metabolites of broiler chicks produced from hens fed diets containing FO or CO for 28 d

| Diet | CO | | FO | | | Effect | | |
|---|---|---|---|---|---|---|---|---|
| Age | 7 d | 14 d | 7 d | 14 d | SEM | Diet | Age | Diet x Age |
| | | | | | | P-value | | |
| Hatch body wt (g) | 41.6 | 41.6 | 40.6 | 38.5 | 1.04 | 0.05 | 0.31 | 0.33 |
| Final body wt (g) | $123^{b}$ | $241^{a}$ | $111^{b}$ | $303^{a}$ | 26.7 | 0.35 | 0.001 | 0.18 |
| Glucose (mg/dL) | 276 | 303 | 346 | 314 | 92.3 | 0.29 | 0.56 | 0.20 |
| NEFA (mM) | 0.57 | 0.65 | 0.61 | 0.64 | 0.27 | 0.85 | 0.49 | 0.80 |
| Abdominal adiposity (%) | $0.34^{c}$ | $1.21^{a}$ | $0.31^{c}$ | $0.75^{b}$ | 0.11 | 0.04 | 0.001 | 0.06 |
| Subcutaneous adiposity (%) | $0.91^{ab}$ | $1.23^{a}$ | $0.85^{b}$ | $0.74^{b}$ | 0.8 | 0.02 | 0.38 | 0.08 |

FO, fish oil; CO, corn oil; N= 10/diet/age; Effect (diet, age, and diet X age) P-value from ANOVA; Labeled means in a row without a common letter differ; $p<0.05$. Adiposity=depot weight/body weight x100

Table 3.5. Differentially expressed proteins in abdominal adipose tissue of FO vs. CO chicks at 14 d

| | FO/CO ratio | P-value[2] | Protein symbol | Protein name |
|---|---|---|---|---|
| Increased (FO/CO) | 4.20 | 0.017 | EPB41L1 | erythrocyte membrane protein band 4.1-like 1 |
| | 3.49 | 0.002 | VIM | Vimentin |
| | 3.24 | 0.023 | HBAA | hemoglobin alpha, subunit A |
| | 3.04 | 0.000 | TBA1 | tubulin alpha-1 chain |
| | 3.01 | 0.024 | ANK1 | ankyrin 1, erythrocytic |
| | 2.97 | 0.034 | NFASC | Neurofascin |
| | 2.71 | 0.001 | DST | Dystonin |
| | 2.69 | 0.001 | FMO3 | dimethylaniline monooxygenase [N-oxide-forming] |
| | 2.69 | 0.000 | ACTG1 | actin, gamma 1 |
| | 2.65 | 0.000 | ACTB | actin, beta |
| | 2.64 | 0.001 | TBB7 | tubulin beta-7 chain |
| | 2.63 | 0.000 | ACT5 | actin, cytoplasmic type 5 |
| | 2.62 | 0.042 | COL28A1 | collagen, type XXVIII, alpha 1 |
| | 2.55 | 0.022 | SPTB | spectrin, beta, erythrocytic |
| | 2.55 | 0.001 | ALB | Albumin |
| | 2.49 | 0.011 | A2ML2 | alpha-2-macroglobulin-like 2 |
| | 2.44 | 0.012 | ITIH2 | inter-alpha-trypsin inhibitor heavy chain 2 |
| | 2.40 | 0.046 | IGF2R | insulin like growth factor 2 receptor |
| | 2.29 | 0.028 | BDH1 | 3-hydroxybutyrate dehydrogenase, type 1 |
| | 2.16 | 0.035 | FBLN1 | fibulin 1 |
| | 2.12 | 0.008 | TUBA3 | tubulin alpha-3 chain-like |
| | 2.01 | 0.042 | PELO | pelota homolog |
| | 2.00 | 0.051 | PLIN1* | perilipin 1 |
| | 1.98 | 0.054 | RAB18* | ras-related protein Rab-18 |
| | 1.88 | 0.017 | RBP4 | retinol-binding protein 4 |
| | 1.85 | 0.017 | ANXA5 | annexin A5 |
| | 1.83 | 0.003 | TMED7 | transmembrane emp24 protein transport domain containing 7 |
| | 1.79 | 0.003 | TF | transferrin |
| | 1.78 | 0.054 | VDAC3 | voltage dependent anion channel 3 |
| | 1.70 | 0.024 | TUBAL3 | tubulin alpha like 3 |
| | 1.69 | 0.002 | TUBB4B | tubulin beta 4B class IVb |
| | 1.69 | 0.007 | TUBA3E | tubulin alpha 3e |
| | 1.65 | 0.010 | TUBA4B | tubulin alpha 4b |
| | 1.63 | 0.024 | PLA2G6 | phospholipase A2 group VI |
| | 1.61 | 0.034 | NSF | N-ethylmaleimide sensitive factor, vesicle fusing ATPase |
| | 1.57 | 0.032 | ERAP1 | endoplasmic reticulum aminopeptidase 1 |
| | 1.54 | 0.034 | DNM1L | dynamin 1 like |

Table 3.5. continued

| | FO/CO ratio | P-value[2] | Protein symbol | Protein name |
|---|---|---|---|---|
| | 1.53 | 0.003 | TBB3 | tubulin beta-3 chain |
| | 1.52 | 0.007 | USP9X | ubiquitin specific peptidase 9, X-linked |
| | 1.50 | 0.043 | PIT54 | PIT54 protein |
| Decreased (FO/CO) | 0.56 | 0.020 | TXN | Thioredoxin |
| | 0.51 | 0.046 | HMGCL | 3-hydroxymethyl-3-methylglutaryl-CoA lyase |
| | 0.45 | 0.019 | PKM | pyruvate kinase, muscle |
| | 0.44 | 0.031 | TNX | avian tenascin X |
| | 0.41 | 0.045 | LAMA5 | laminin, alpha 5 |
| | 0.38 | 0.024 | PPA2 | pyrophosphatase |
| | 0.33 | 0.008 | FXR1 | fragile X mental retardation, autosomal homolog 1 |
| | 0.33 | 0.034 | GIF | gastric intrinsic factor |
| | 0.31 | 0.033 | NT5C2 | 5'-nucleotidase, cytosolic II |
| | 0.31 | 0.033 | NT5C3 | cytosolic purine 5'-nucleotidase |
| | 0.31 | 0.014 | FBP1 | fructose-1,6-bisphosphatase 1 |
| | 0.28 | 0.050 | UQCRFS1 | ubiquinol-cytochrome c reductase, Rieske iron-sulfur polypeptide 1 |
| | 0.25 | 0.039 | LBFABP | liver basic fatty acid binding protein |
| | 0.22 | 0.032 | ATP2A3 | ATPase, Ca++ transporting, ubiquitous |
| | 0.21 | 0.008 | MYH1G | myosin, heavy chain 1G, skeletal muscle |
| | 0.20 | 0.009 | CKM | creatine kinase, muscle |
| | 0.20 | 0.009 | MYH1C | myosin, heavy chain 1C, skeletal muscle |
| | 0.20 | 0.006 | MYL1 | myosin, light chain 1, alkali; skeletal, fast |
| | 0.19 | 0.021 | PCK2 | phosphoenolpyruvate carboxykinase 2 |
| | 0.18 | 0.004 | MYH1B | myosin, heavy chain 1B, skeletal muscle |
| | 0.17 | 0.003 | MYH1F | myosin, heavy chain 1F, skeletal muscle |
| | 0.17 | 0.020 | MYL3 | myosin, light chain 3, alkali; ventricular, skeletal, slow |
| | 0.17 | 0.006 | CRYAB | crystallin, alpha B |
| | 0.17 | 0.005 | MYSS | myosin heavy chain, skeletal muscle |
| | 0.15 | 0.005 | MYH1E | myosin, heavy chain 1E, skeletal muscle |
| | 0.15 | 0.003 | MYH1D | myosin, heavy chain 1D, skeletal muscle |
| | 0.15 | 0.021 | PDLIM5 | PDZ and LIM domain 5 |
| | 0.15 | 0.004 | ENO3 | enolase 3 |
| | 0.15 | 0.002 | MYLPF | myosin light chain, phosphorylatable, fast skeletal muscle |
| | 0.15 | 0.013 | AK1 | adenylate kinase 1 |
| | 0.15 | 0.002 | MYH1A | myosin, heavy chain 1A, skeletal muscle |
| | 0.14 | 0.007 | DMD | Dystrophin |
| | 0.13 | 0.002 | LAMA2 | laminin, alpha 2 |

Table 3.5. continued

| FO/CO ratio | P-value[2] | Protein symbol | Protein name |
|---|---|---|---|
| 0.13 | 0.003 | GATM | glycine amidinotransferase |
| 0.13 | 0.002 | FABP1 | fatty acid binding protein 1 |
| 0.12 | 0.000 | ANKRD2 | ankyrin repeat domain 2 |
| 0.11 | 0.003 | MYH13 | myosin, heavy chain 13, skeletal muscle |
| 0.11 | 0.004 | MYH15 | myosin, heavy chain 15 |
| 0.10 | 0.000 | MYL2 | myosin, light chain 2, regulatory, cardiac, slow |
| 0.10 | 0.003 | MYSC | myosin heavy chain, cardiac muscle isoform |
| 0.10 | 0.000 | ADPRHL1 | ADP-ribosylhydrolase like 1 |
| 0.10 | 0.007 | MYOZ2 | myozenin 2 |
| 0.10 | 0.000 | FBP2 | fructose-1,6-bisphosphatase 2 |
| 0.09 | 0.027 | SRCA | sarcalumenin |
| 0.09 | 0.002 | XIRP1 | xin actin-binding repeat containing 1 |
| 0.09 | 0.002 | ATP2A1 | ATPase, Ca++ transporting, cardiac muscle, fast twitch 1 |
| 0.08 | 0.000 | TMOD4 | tropomodulin 4 |
| 0.08 | 0.022 | TNNI2 | troponin I type 2 |
| 0.08 | 0.001 | AMPD1 | adenosine monophosphate deaminase 1 |
| 0.06 | 0.052 | MYOM1 | myomesin 1 |
| 0.06 | 0.020 | APOBEC2 | apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like 2 |
| 0.05 | 0.010 | CAMSAP1 | calmodulin regulated spectrin associated protein 1 |
| 0.04 | 0.003 | TNNT3 | troponin T type 3 |
| 0.04 | 0.004 | CASQ2 | calsequestrin 2 |
| 0.04 | 0.009 | MYOZ1 | myozenin 1 |

FO/CO ratio is the ratio of normalized protein abundance in abdominal adipose tissue at 14 d from N=3 samples per diet group; CO, corn oil; FO, fish oil. P-values are from T-tests, FO vs. CO

* included to show trend because of potential biological relevance

Table 3.6. Functional enrichment of differentially abundant proteins in adipose tissue of chicks produced from hens fed diets containing FO or CO for 28 d

| GO Enrichment | | | |
|---|---|---|---|
| ID | Term | Count | Adj. P-value |
| GO:0007010 | cytoskeleton organization | 11 | $1.16 \times 10^{-6}$ |
| GO:0030049 | muscle filament sliding | 7 | $3.85 \times 10^{-6}$ |
| GO:0006094 | gluconeogenesis | 5 | $3.49 \times 10^{-3}$ |
| GO:0007517 | muscle organ development | 6 | $3.31 \times 10^{-3}$ |
| GO:0007017 | microtubule-based process | 4 | $3.26 \times 10^{-2}$ |
| GO:0006928 | movement of cell or subcellular component | 5 | $3.26 \times 10^{-2}$ |
| KEGG Pathway | | | |
| ID | Pathway | Count | Adj. P-value |
| 04530 | Tight junction | 7 | 0.0052 |
| 00010 | Glycolysis/gluconeogenesis | 5 | 0.0297 |

FO, fish oil; CO, corn oil; N=6/diet/age; Adj. P-value = P-value adjusted for false discovery using Bonferroni correction.

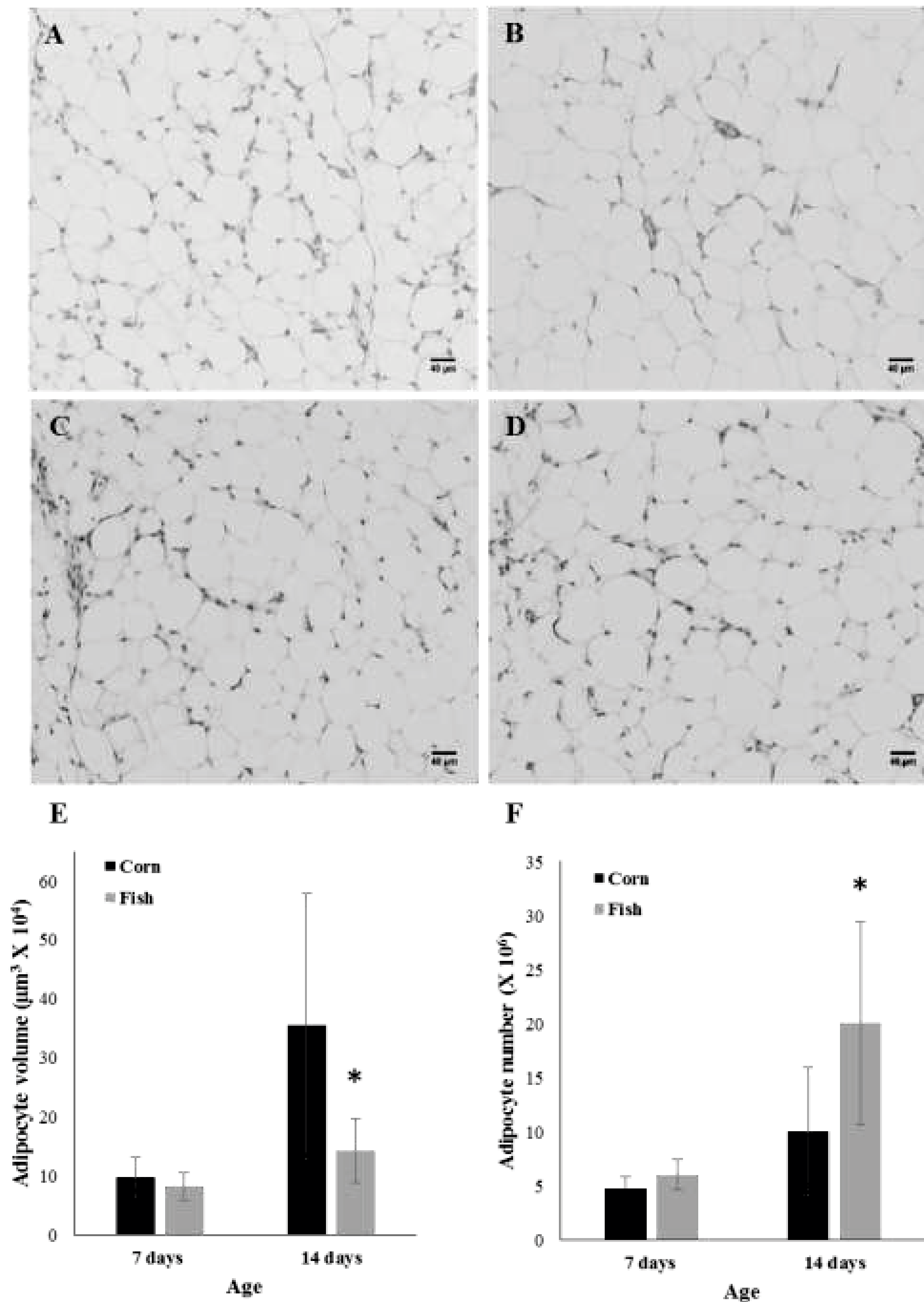


Figure 3.1. Adipocyte volume and number in CO and FO chicks at 7 and 14 d of age. Representative H&E-stained images of abdominal adipose tissue from CO (A, B) and FO (C, D) used to determine adipocyte volume at 7 (A, C) and 14 (B, D) d. Scale bar = 40 µm. Two slides and three independent fields/slide were counted in three chicks in each age/diet group. (E), average adipocyte volume ($\mu m^3$ X $10^4$), ± SD; (F) average adipocyte number (X $10^6$),± SD; * = P<0.05, FO vs. CO, post-hoc comparison using LSM

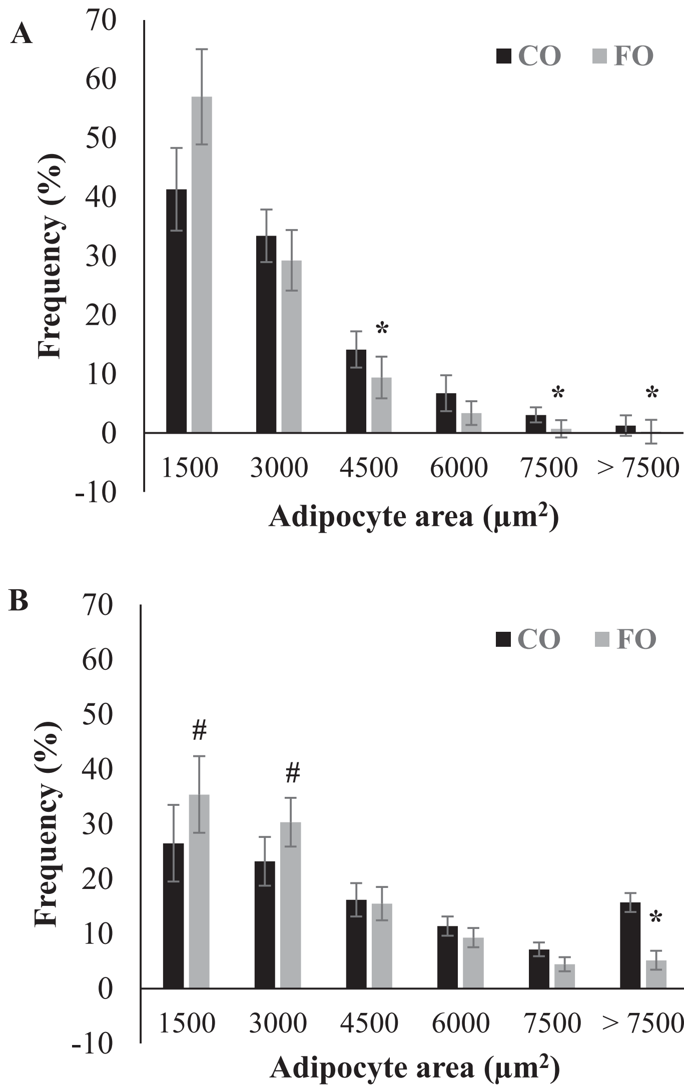


Figure 3.2 Frequency distribution of adipocyte area (µm$^2$) of CO and FO chicks at 7 (A) and 14 (B) d of age. Adipocyte areas measured from H&E-stained images were divided into arbitrary bins; average frequency of cells within each bin, ± SEM; * = $P \leq 0.05$; # = $P \leq 0.10$, FO vs. CO.

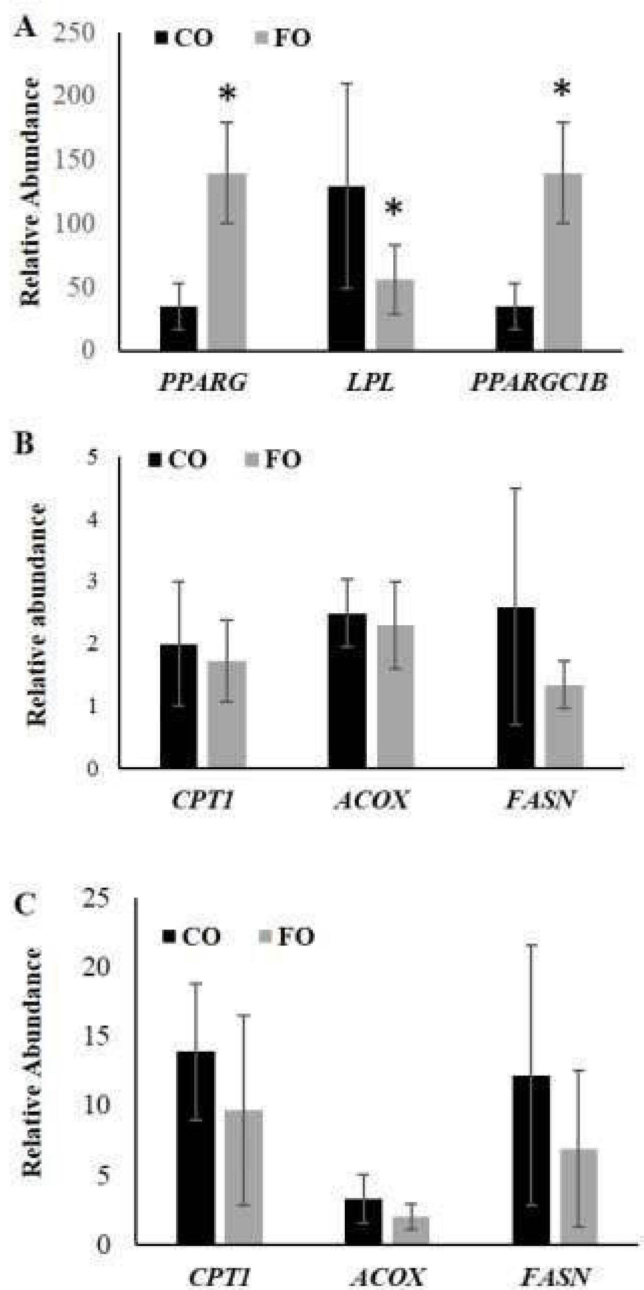


Figure 3.3. Expression of genes involved in adipogenesis, fatty acid oxidation and uptake of fatty acids in adipose tissue (A, B) and liver (C) of CO and FO at two weeks of age.ACOX1, Acyl-CoA oxidase 1; CPT1, Carnitine palmitoyltransferase 1; PPARG, Peroxisome proliferator-activated receptor gamma; PPARGC1B, Peroxisome proliferator-activated receptor gamma co-activator 1 Beta; FASN, Fatty acid synthase; LPL, Lipoprotein lipase; average relative abundance, ± SEM;* = $P \leq 0.05$, FO vs. CO; n=5-6/diet group.

# CHAPTER IV.

# TRANSCRIPTIONAL ANALYSIS OF ABDOMINAL FAT OF BROILER CHICKS PROGRAMMED TOWARDS REDUCED FAT DEPOSITION

A version of this manuscript is being prepared for publication by: Ronique C. Beckford, Matthew Huff, Margaret. Staton, Robert. I. Mihelic, Suchita Das, Brynn H. Voy. RCB performed the experiments, analyzed data and drafted the manuscript. MH and MS assisted with RNA sequencing analysis. BHV directed the project, obtained funding, and reviewed and edited the manuscript and is the corresponding author.

## Abstract

Adipose tissue is subject to maternal programing, in which the maternal diet or environment before birth influences adipose accumulation in offspring. Dietary long chain n-3 polyunsaturated fatty acids (LC n-3 PUFA) have been shown to reduce adipose mass in broiler chickens when fed during the period of rapid growth between hatch and market age. We recently demonstrated that providing LC n-3 PUFA in the hen diet reduced adiposity in chicks compared to a diet enriched in n-6 PUFA, suggesting the potential to program reduced adiposity in chicks through the type of fat provided to the hen. The objective of this study was to identify potential mechanisms for LC n-3 PUFA programming of adipose deposition. Abdominal adipose tissue of chicks that hatched from hens fed fish oil (LC n-3 PUFA) or corn oil (n-6 PUFA) was profiled by RNA sequencing (RNAseq). Cobb 500 broiler chicks were hatched from broiler breeder hens fed diets containing fat (5%) from either fish oil (FO) or corn oil (CO) for 4 weeks. Post hatch, chicks were fed a CO-based diet after hatch, until 7 or 14 days of age. The type of fat provided in the hen diet significantly affected chick adipose transcriptomes at both 7d and 14 d. At 7 d 190 genes were upregulated while 46 genes were downregulated in FO vs. CO chicks. The influence of hen diet persisted at 14 d, although fewer genes (45) were significantly affected. Hen FO feeding was associated with differential expression of genes involved in mesenchymal stem cell differentiation and muscle development at 7d, as well as a number of genes involved in lipid and glucose metabolism. Although fewer genes were affected by diet at 14 d, effects on lipid metabolism and

other aspects of cell development were apparent. A total of eight genes were differentially expressed at both ages, indicating that part of the influence of diet is sustained after hatch. In conclusion, these data suggest several candidate pathways that may contribute to programming of adipose development through maternal dietary fat.

## Introduction

Two major goals of the poultry industry are to increase carcass yield and reduce the amount of fat deposited in the abdomen (Fouad, et al., 2014). However, broilers selected for their fast growth and efficient feed conversion inadvertently deposit fat in their abdomen (Griffin, et al., 1992). Ultimately, excess fat deposition wastes feed, reduces feed efficiency, and increases processing cost. Hence reducing is the amount of fat accumulated here would be economically beneficial to producers. The abdominal fat depot differs from other depots in both size and time of development. Unlike the neck and subcutaneous depot, abdominal fat develops significantly after hatch (Butterwith, 1997). Also, the growth of the abdominal fat pad occurs through both hyperplasia and hypertrophy until the chicks are approximately two weeks of age, after which hypertrophy is responsible for its expansion (Butterwith, 1997; Hood, 1982). Since there are two distinct time points and mechanisms by which abdominal fat accumulates, multiple opportunities to target excess fat deposition are possible.

Various studies have shown that dietary manipulations may be a way to reduce excess fat deposition in broilers. One such manipulation that has been utilized is altering the different fatty acids included in the diet. Fatty acids, particularly long chain n-3 polyunsaturated fatty acids (LC n-3 PUFA) have been found to reduce excess fat deposition in rodents, humans, and chickens. According to Azain, (2004) fatty acids and their derivatives may have hormone-like effects and have been be shown to regulate gene expression in preadipocytes, which ultimately effects their proliferation and differentiation. However few studies have evaluated the impact of providing

these nutrients during development. Developmental programming refers to the concept that, early exposure of the developing embryo to certain stimuli influences growth and development. This impact can have detrimental consequences not only on the offspring but also future generations (Elshenawy, et al., 2016). Studies in humans have shown that children exposed to chemicals such as bisphenol A (Veiga-Lopez, et al., 2016), air pollutants (Lavigne, et al., 2016), or cigarette smoke (Timmermans, et al., 2014) before birth may express physiological changes during development.

In the current study we capitalized on the relationship between the diet a chicken consumes and the impact it has on the egg. Unlike in ruminants, fatty acids found in the diet are not altered during digestion. Therefore, the fatty acid content of the egg is a direct reflection of the hen diet. Hence, the developing embryo will have access to these fatty acids which will directly influence their development. Utilizing this model, we previously demonstrated that early exposure of the developing embryo to the LC n-3 PUFA eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) attenuated adipose mass after hatch without impacting growth. Chicks hatched from hens that were fed a diet enriched in fish oil (FO; 5%) had reduced adipose weight, with smaller adipocytes, than those hatched from hens fed equal amounts of fat as corn oil (CO). All chicks consumed a CO-based diet after hatch, suggesting that reduced adiposity resulted from developmental programming through hen dietary fat source. Proteomic analysis of adipose tissue from 14d chicks indicated that gluconeogenesis and adipocyte cytoskeleton were influenced by the hen diet. Understanding the mechanisms through which maternal dietary fat quality regulates offspring adipose development has important potential applications for both the poultry industry and for human health. Therefore in the present study we utilized RNA sequencing to compare the

adipose transcriptomes of chicks that were hatched from hens fed FO or CO to identify potential mechanisms through which dietary fat programs offspring adipose development.

## Materials and Methods

### Tissue collection and RNA extraction

Detailed animal husbandry procedures can be found in (Beckford, et al., 2017). At dissection, abdominal adipose tissue samples were snap-frozen in liquid nitrogen and stored at –80$^{o}$C. Total RNA used for sequencing was isolated from abdominal adipose tissue of five chicks in each treatment group using TRIzol$^{TM}$ (Invitrogen, Carlsbad, CA). Frozen abdominal adipose tissue was first pulverized under liquid nitrogen to produce a homogeneous powdered sample, from which approximately 200 mg were removed for RNA isolation. Quality and quantity of extracted RNA samples were measured using the Experion (Bio-Rad (Hercules, CA)) automated electrophoresis system. RNA quality indicator (RQI) values of samples ranged from 7.4 to 9.8.

### RNA sequencing

RNA sequencing was conducted by Novogene (Chula Vista, CA) using the Illumina HiSeq 4000 sequencing platform (Illumina, San Diego, CA). After independent verification of RNA quality, cDNA libraries were created from each RNA sample (5/diet/age; n=20) using NEBNext Ultra RNA Library kit according to procedures developed by the manufacturer (Illumina). After library construction, the insert size of each library was determined using a 2100 Bioanalyzer (Agilent, Santa Clara, CA). QPCR was used to quantify effective concentration (> 2 nM) of each library prior to sequencing. A minimum of 20 M PE 150 bp reads were obtained from each library. Quality of sequencing from each library was evaluated using fastQC before proceeding with statistical analyses http://www.bioinformatics.babraham.ac.uk/projects/fastqc/.

### Trimming and mapping

The reference genome for the chicken (*Gallus gallus*, build 5.0) in FASTA format and the corresponding annotation file in GTF format were obtained from Ensembl (ftp.ensembl.org/pub/release–90). This version of the chicken genome consists of 18,346 genes, represented by 38,118 transcripts. Prior to mapping, reads were trimmed using skewer with default parameters (Jiang, et al., 2014) to remove adapters. Reads were mapped to the chicken genome using STAR 2.4.0.1 (Dobin, et al., 2013). The generated BAM alignment files were then converted to SAM files using SAM tools and a table of read counts was generated using HTSeq-count (http://www-huber.embl.de/users/anders/HTSeq/doc/overview.html) for analysis of differential expression.

### Differential gene expression

The R statistical computing package (v. 3. 4. 2), DESeq2 (v 1.16.1) was used to identify genes that were differentially expressed in FO vs. CO chick adipose tissue. DESeq2 uses as input a matrix of un-normalized read counts, which are internally corrected for variation in library size. Pre-filtering was performed by excluding transcripts with fewer than 10 total reads across the 10 samples (5 FO and 5 CO) in each age group. Statistically significant effects of diet on gene expression in each of the two age groups were identified using t-test, using p-values adjusted for FDR ($\alpha = 0.05$). The Database for Annotation, Visualization and Integrated Discovery (DAVID), v 6.8, was used to functionally annotate differentially expressed genes based on Gene Ontology (GO) annotation and KEGG pathway enrichment (Huang, et al., 2008).

## Results

RNA sequencing was used to characterize the abdominal adipose tissue gene expression of broiler chicks hatched from hens fed either a diet containing CO or FO for four weeks. A total of 20 – 56 million reads were produced for each sample. Approximately 70% of reads were mapped

to the genome, representing ~ 18,000 genes (Table 4.1). The extent to which abdominal gene expression profiles could discriminate chicks based on maternal diet was evaluated using unsupervised clustering. As shown in Fig. 4.1, chicks separated into their treatment groups, based on expression of the 50 most variable genes between samples. These results illustrate the relative effects of the maternal diet on chick adipose transcriptomes. Comparison of FO and CO at each of the two ages indicated that maternal dietary fat source significantly affected abdominal adipose tissue gene expression post-hatch. A total of 236 genes differed significantly (adj. p-value $\leq 0.05$) between the two groups at 7 d .Of these, 136 transcripts are annotated to known or putative genes in the ENSEMBL database, based on the current assembly (Gallus_gallus-5.0) of the chicken genome. A total of 190 genes were upregulated, and 36 downregulated in FO relative to CO chicks (Table 4.2) (Table 4.3). Functional analysis based on GO annotation revealed that FO increased the expression of a set of genes (~ 23) that are assigned functions related to muscle cell differentiation and development. In addition, expression of several genes related to lipid and glucose metabolism (e.g., fatty acid binding protein 1 (FABP1), liver basic fatty acid binding protein (LBFABP), fructose-1,6-bisphosphatase 2 (FBP2), protein kinase, AMP-activated, alpha 2 catalytic subunit (PRKAA2) were upregulated in FO vs. CO adipose tissue at this age. The set of 36 genes that were downregulated in FO vs. CO included glucagon (GCG), glucagon receptor-like protein (LOC772096), insulin like growth factor 2 receptor (IGF2R) and other genes with various functional annotations. Effects of the maternal diet on chick adipose transcriptomes persisted at 14 d, although the number of genes that differed significantly between the two groups (45) was lower than at 7 d. A total of 32 genes were upregulated (Table 4.4) and 13 downregulated (Table 4.5), in FO vs. CO at this age. Effects of diet on lipid metabolism were reflected in persistent upregulation of FABP1, and by down-regulation of stearoyl-CoA desaturase 5, in FO vs. CO

adipose tissue. Eight genes (two of which are unannotated) were significantly affected by diet at both ages.

## Discussion

Diet is the most easily manipulated component of poultry production, and the intuitive choice for strategies to reduce fat deposition. However, modifying the diet can be costly. Even modest increases to the cost of a ration can have significant impact because feed is the primary cost of production, and broilers eat ad libitum. Further, diet modifications cannot compromise growth. Adipose tissue is known to be influenced by developmental programming, through which the diet and lifestyle of the mother alter the accumulation of adipose tissue in offspring. In humans, the relationship between maternal diet and lifestyle and child adipose tissue can persist years after birth, into adulthood. With respect to poultry production, this potential for programming of adipose tissue creates the possibility to exert sustained impact on fat accretion by altering the maternal diet while still feeding a standard ration in chicks after hatch. We recently demonstrated that feeding hens a diet enriched in FO reduces adipose mass and alters adipocyte development in chicks, compared to a diet enrich in CO. Understanding the potential mechanisms for this response has broad utility for poultry and other species, and provided the rational for transcriptomic analyses described herein.

A number of genes with functions relevant to adiposity were differentially expressed between FO and CO chicks at 7d. Two intracellular fatty acid binding proteins (fatty acid binding protein 1 (FABP1) and liver basic fatty acid binding protein (LBFABP)) were upregulated in FO chick adipose tissue. Fatty acid binding protein 1 is involved in lipid uptake, and its expression is induced during proliferation of adipocytes, as differentiation is initiated (Samulin, et al., 2008). The function of LBFABP is not been well-characterized, although it was shown to be differentially expressed in liver of genetically lean vs. fat chickens (Zhang, et al., 2013). No orthologs have been

described in mouse or human, suggesting that it may be a novel avian fatty acid binding protein. Potential effects of maternal diet on lipid handling are also reflected by expression of α/β hydrolase fold domain 3 (ABHD3), which was downregulated in FO vs. CO tissue. This gene is one of a family of lipid metabolizing proteins that has been suggested to play an important signaling role in the control of energy balance (Lord, et al., 2013). Expression of ABHD3 is regulated by PPARγ, which is considered as the master transcriptional regulator of adipogenesis (Soccio, et al., 2015). At least three genes that differed between FO and CO at 7 d regulate signaling activity through AMPK and protein kinase A (PKA), both of which influence lipid metabolism in adipocytes. Adenosine monophosphate deaminase 1 (AMPD1) controls the phosphorylation status of AMPK (Tandelilin, et al., 2015), a subunit of which is encoded by protein kinase, AMP-activated, alpha 2 catalytic subunit (PRKAA2). Both were expressed at significantly higher levels in FO chick adipose tissue.

One possible mechanism through which hen dietary fish oil altered chick adipose gene expression after hatch is through epigenetic modification. Methylation status of several of the genes affected by maternal diet at 7d has been associated with adiposity in other species. The purinergic receptor P2RY1 regulates leptin production in white adipocyte (Laplante, et al., 2010)s, and its methylation level correlates with body mass index in humans (Ramos-Lopez, et al., 2018). Methylation of phosphoglycerate dehydrogenase (PHGDH), which is involved in serine biosynthesis, has also been associated with BMI and with abdominal adiposity in large-scale human studies (Aslibekyan, et al., 2015). The maternal diet has been shown to specifically alter expression levels and methylation of insulin-like growth factor receptor 2 (IGFR2), PRKAA2, and ankyrin repeat domain 1 (ANKRD1) in fetal adipose tissue. have been shown to be differentially methylated in fetal adipose tissue in response to maternal diet (Lan, et al., 2013). Epigenetic

modification is well-described for IGFR2, which regulates adipocyte proliferation and differentiation in developing adipose tissue (Holly, et al., 2006).

In conclusion, understanding the molecular mechanisms of fat deposition and how they can be regulated is necessary in order to reduce excess accumulation (Resnyk, et al., 2015). In chickens, both hyperplasia and hypertrophy play key roles in adipose deposition. These two mechanisms exert dominance at two different time points with an overlap occurring before hatch and two weeks post hatch. This study highlights genes in potential pathways that may be regulated by the maternal diet enriched in n-3 LCPUFA.

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## Appendix

Table 4.1. Summary of reads mapped from RNA Sequencing of abdominal adipose tissue of FO and CO chick at 7 and 14 d

| Treatment | Age (d) | Assigned | Ambiguous | Alignment not Unique | No feature |
|---|---|---|---|---|---|
| CO | 7 | 19959346 | 781604.4 | 2623009 | 5017301 |
| FO | 7 | 15356491 | 616266.8 | 2083788 | 3894141 |
| CO | 14 | 18652824 | 827766.8 | 2541828 | 4416683 |
| FO | 14 | 18558560 | 710662.2 | 2305025 | 4628521 |

Table 4.2. Genes upregulated in abdominal adipose tissue of FO vs. CO chicks at 7 d

| ENSEMBL ID | Gene name | Fold change (FO/CO) | p. adj. |
|---|---|---|---|
| ENSGALG00000004044 | cysteine and glycine-rich protein 3 | 217.66 | 0.001 |
| ENSGALG00000006025 | xin actin-binding repeat containing 1 | 162.69 | 0.001 |
| ENSGALG00000002988 | phosphoglycerate dehydrogenase | 2.15 | 0.001 |
| ENSGALG00000016300 | tubulointerstitial nephritis antigen | 93.60 | 0.001 |
| ENSGALG00000008914 | nebulin-related anchoring protein | 147.31 | 0.001 |
| ENSGALG00000015602 | creatine kinase, mitochondrial 2 | 223.43 | 0.001 |
| ENSGALG00000015018 | calsequestrin 2 (cardiac muscle) | 185.06 | 0.001 |
| ENSGALG00000011086 | actin, alpha 1, skeletal muscle | 148.68 | 0.001 |
| ENSGALG00000014463 | actinin, alpha 2 | 129.51 | 0.001 |
| ENSGALG00000007539 | ankyrin repeat domain 2 | 83.97 | 0.001 |
| ENSGALG00000018946 | leiomodin 3 (fetal) | 147.00 | 0.001 |
| ENSGALG00000012783 | myosin binding protein C, slow type | 168.90 | 0.001 |
| ENSGALG00000006273 | myosin light chain kinase 2 | 167.51 | 0.001 |
| ENSGALG00000011991 | myozenin 2 | 129.35 | 0.002 |
| ENSGALG00000026594 | heparan sulfate (glucosamine) 3-O-sulfotransferase 5 | 2.82 | 0.002 |
| ENSGALG00000006591 | troponin I type 2 (skeletal, fast) | 96.16 | 0.002 |
| ENSGALG00000002907 | myosin, light chain 1, alkali; skeletal, fast | 155.52 | 0.002 |
| ENSGALG00000009844 | actin, alpha, cardiac muscle 1 | 123.39 | 0.002 |
| ENSGALG00000005226 | myozenin 1 | 104.93 | 0.002 |
| ENSGALG00000005843 | eukaryotic translation elongation factor 1 alpha 2 | 80.31 | 0.002 |
| ENSGALG00000027771 | voltage-dependent calcium channel gamma-1 subunit-like | 150.26 | 0.002 |
| ENSGALG00000005613 | paired-like homeodomain 3 | 97.82 | 0.002 |
| ENSGALG00000001793 | myosin, light chain 10, regulatory | 142.76 | 0.002 |
| ENSGALG00000016309 | kelch like family member 31 | 251.16 | 0.002 |
| ENSGALG00000005448 | myosin, light chain 3, alkali; ventricular, skeletal, slow | 121.39 | 0.002 |
| ENSGALG00000019157 | small muscle protein, X-linked | 177.49 | 0.003 |
| ENSGALG00000013634 | leucine rich repeat containing 2 | 124.96 | 0.003 |
| ENSGALG00000014847 | myomesin 1 | 41.55 | 0.003 |
| ENSGALG00000006572 | troponin T type 3 (skeletal, fast) | 52.17 | 0.003 |
| ENSGALG00000026452 | muscle related coiled-coil protein | 109.01 | 0.004 |
| ENSGALG00000002597 | MSS51 mitochondrial translational activator | 11.89 | 0.005 |
| ENSGALG00000002186 | unc-45 homolog B (C. elegans) | 193.17 | 0.005 |
| ENSGALG00000005292 | kelch-like family member 40 | 61.73 | 0.005 |
| ENSGALG00000021340 | carbonic anhydrase IX | 9.12 | 0.005 |

Table 4.2. continued

| ENSEMBL ID | Gene name | Fold change (FO/CO) | p. adj. |
|---|---|---|---|
| ENSGALG00000010357 | purinergic receptor P2Y1 | 4.98 | 0.005 |
| ENSGALG00000028871 | solute carrier family 38, member 3 | 36.42 | 0.006 |
| ENSGALG00000015935 | SET and MYND domain containing 1 | 17.31 | 0.007 |
| ENSGALG00000012612 | fructose-1,6-bisphosphatase 2 | 34.65 | 0.007 |
| ENSGALG00000016127 | SH3 domain binding glutamate rich protein | 57.84 | 0.009 |
| ENSGALG00000006835 | troponin C type 2 | 99.45 | 0.009 |
| ENSGALG00000023435 | glycine amidinotransferase | 35.76 | 0.009 |
| ENSGALG00000006216 | myogenic differentiation 1 | 76.37 | 0.009 |
| ENSGALG00000023772 | heat shock 27kDa protein family, member 7 | 32.57 | 0.009 |
| ENSGALG00000002125 | tropomodulin 1 | 24.64 | 0.009 |
| ENSGALG00000005297 | hedgehog acyltransferase-like | 21.91 | 0.009 |
| ENSGALG00000026536 | inositol hexakisphosphate kinase 3 | 121.46 | 0.009 |
| ENSGALG00000026875 | uncharacterized LOC428186 | 42.82 | 0.009 |
| ENSGALG00000002082 | adenosine monophosphate deaminase 1 | 32.81 | 0.009 |
| ENSGALG00000005754 | dual specificity protein phosphatase 22-A-like | 27.92 | 0.009 |
| ENSGALG00000005493 | integrin beta 1 binding protein (melusin) 2 | 26.11 | 0.009 |
| ENSGALG00000007335 | kelch repeat and BTB domain containing 13 | 8.81 | 0.009 |
| ENSGALG00000011094 | phosphodiesterase 4B, cAMP-specific | 1.93 | 0.009 |
| ENSGALG00000008226 | NGG1 interacting factor 3 like 1 | 1.64 | 0.009 |
| ENSGALG00000008905 | hyaluronan binding protein 2 | 17.80 | 0.009 |
| ENSGALG00000005621 | calcium/calmodulin-dependent protein kinase II alpha | 77.93 | 0.010 |
| ENSGALG00000001459 | troponin C type 1 | 36.92 | 0.010 |
| ENSGALG00000016826 | ADP-ribosylhydrolase like 1 | 53.72 | 0.010 |
| ENSGALG00000009262 | fibrinogen beta chain | 4.38 | 0.011 |
| ENSGALG00000006491 | ankyrin repeat domain 1 (cardiac muscle) | 41.76 | 0.012 |
| ENSGALG00000004141 | liver basic fatty acid binding protein | 4.12 | 0.012 |
| ENSGALG00000004582 | myosin, light chain 2, regulatory, cardiac, slow | 146.95 | 0.012 |
| ENSGALG00000008805 | leiomodin 2 | 140.89 | 0.012 |
| ENSGALG00000028949 | coronin 6 | 47.86 | 0.012 |
| ENSGALG00000000164 | myosin binding protein H | 185.10 | 0.012 |
| ENSGALG00000004357 | annexin A6 | 1.25 | 0.012 |
| ENSGALG00000006819 | ATP/GTP binding protein-like 1 | 32.14 | 0.013 |

Table 4.2. continued

| ENSEMBL ID | Gene name | Fold change (FO/CO) | p. adj. |
|---|---|---|---|
| ENSGALG00000006508 | fibroblast growth factor 13 | 30.74 | 0.016 |
| ENSGALG00000006346 | C-X-C motif chemokine ligand 14 | 5.22 | 0.016 |
| ENSGALG00000006008 | homer scaffolding protein 2 | 7.21 | 0.016 |
| ENSGALG00000008100 | receptor associated protein of the synapse | 8.55 | 0.016 |
| ENSGALG00000009435 | protein phosphatase 1, regulatory subunit 3A | 198.82 | 0.017 |
| ENSGALG00000007945 | crystallin, alpha B | 20.30 | 0.017 |
| ENSGALG00000016859 | methyltransferase like 21E, pseudogene | 23.02 | 0.018 |
| ENSGALG00000009012 | zinc finger protein 385B | 9.63 | 0.019 |
| ENSGALG00000017316 | uncoupling protein 3 | 15.03 | 0.020 |
| ENSGALG00000005537 | ankyrin repeat and SOCS box containing 14 | 16.04 | 0.020 |
| ENSGALG00000010614 | solute carrier family 25 | 13.52 | 0.025 |
| ENSGALG00000019845 | avian beta-defensin 9 | 5.29 | 0.026 |
| ENSGALG00000003925 | Myopalladin | 18.95 | 0.027 |
| ENSGALG00000009082 | aarF domain containing kinase 3 | 2.71 | 0.030 |
| ENSGALG00000013056 | tubulin, alpha 8a | 104.77 | 0.031 |
| ENSGALG00000009523 | contactin 1 | 2.31 | 0.033 |
| ENSGALG00000014695 | chloride channel, voltage-sensitive 1 | 7.41 | 0.034 |
| ENSGALG00000029150 | adenylate kinase 1 | 10.10 | 0.034 |
| ENSGALG00000001626 | urotensin 2 receptor | 9.99 | 0.035 |
| ENSGALG00000026395 | TraB domain containing 2A | 5.89 | 0.037 |
| ENSGALG00000009682 | ventricular zone expressed PH domain containing 1 | 4.42 | 0.037 |
| ENSGALG00000006300 | lactate dehydrogenase A | 7.42 | 0.037 |
| ENSGALG00000015410 | blood vessel epicardial substance | 43.97 | 0.038 |
| ENSGALG00000005019 | dual specificity phosphatase and pro isomerase domain containing 1 | 16.20 | 0.039 |
| ENSGALG00000011324 | tubulin alpha 4b | 3.45 | 0.041 |
| ENSGALG00000012010 | N-deacetylase/N-sulfotransferase | 3.11 | 0.044 |
| ENSGALG00000004840 | rabphilin 3A homolog | 2.55 | 0.044 |
| ENSGALG00000007594 | ankyrin repeat and SOCS box containing 12 | 4.13 | 0.044 |
| ENSGALG00000006260 | kelch like family member 30 | 68.27 | 0.045 |
| ENSGALG00000011447 | glutamate decarboxylase-like 1 | 15.13 | 0.045 |
| ENSGALG00000012845 | myosin light chain kinase family, member 4 | 56.79 | 0.046 |
| ENSGALG00000000107 | tripartite motif containing 7.1 | 7.15 | 0.046 |

Table 4.2. continued

| ENSEMBL ID | Gene name | Fold change (FO/CO) | p. adj. |
|---|---|---|---|
| ENSGALG00000016342 | myomesin 2 | 32.44 | 0.046 |
| ENSGALG00000013822 | taxilin beta | 12.35 | 0.046 |
| ENSGALG00000003816 | 5'-nucleotidase, cytosolic IA | 10.99 | 0.046 |
| ENSGALG00000015937 | fatty acid binding protein 1 | 4.81 | 0.046 |
| ENSGALG00000002728 | solute carrier family 16 | 4.96 | 0.047 |
| ENSGALG00000012964 | ANKH inorganic pyrophosphate transport regulator | 1.54 | 0.048 |
| ENSGALG00000010826 | protein kinase, AMP-activated, alpha 2 catalytic subunit | 5.93 | 0.048 |
| ENSGALG00000009446 | leucine-rich single-pass membrane protein 1 | 7.37 | 0.048 |
| ENSGALG00000010881 | ankyrin repeat and SOCS box containing 2 | 5.81 | 0.053 |
| ENSGALG00000027090 | uncharacterized LOC422926 | 4.66 | 0.053 |
| ENSGALG00000009718 | family with sequence similarity 53 member B | 1.28 | 0.053 |
| ENSGALG00000005972 | kelch repeat and BTB domain containing 12 | 28.92 | 0.055 |

Upregulated genes in abdominal adipose tissue at 7 d from N=5 samples per diet group; CO, corn oil; FO, fish oil. Adj. P-values are from T-tests, FO vs. CO. Genes represents those that were annotated in the chicken genome (*Gallus gallus* 5.0)

Table 4.3. Genes downregulated in abdominal adipose tissue of FO vs. CO chicks at 7 d

| ENSEMBL ID | Gene name | Fold change (FO/CO) | p. adj. |
|---|---|---|---|
| ENSGALG00000004621 | phosphatidylinositol-3,4,5-trisphosphate-dependent Rac exchange factor 1 | 0.58 | 0.001 |
| ENSGALG00000021345 | UDP-GlcNAc:betaGal beta-1,3-N-acetylglucosaminyltransferase 9 | 0.65 | 0.002 |
| ENSGALG00000028895 | glucagon receptor-like | 0.31 | 0.002 |
| ENSGALG00000010046 | family with sequence similarity 177, member A1 | 0.79 | 0.003 |
| ENSGALG00000009040 | TBC1 domain family, member 8B (with GRAM domain) | 0.72 | 0.005 |
| ENSGALG00000009158 | CDC42 binding protein kinase alpha (DMPK-like) | 0.55 | 0.006 |
| ENSGALG00000014236 | ATPase, aminophospholipid transporter (APLT), class I, type 8A, member 1 | 0.43 | 0.007 |
| ENSGALG00000017405 | natriuretic peptide receptor 3 | 0.45 | 0.008 |
| ENSGALG00000014537 | Bcl2 modifying factor | 0.67 | 0.009 |
| ENSGALG00000005914 | family with sequence similarity 21, member A | 0.69 | 0.010 |
| ENSGALG00000011104 | Glucagon | 0.65 | 0.010 |
| ENSGALG00000014938 | abhydrolase domain containing 3 | 0.47 | 0.011 |
| ENSGALG00000000723 | syntaxin 12 | 0.80 | 0.015 |
| ENSGALG00000011241 | raftlin, lipid raft linker 1 | 0.65 | 0.016 |
| ENSGALG00000011533 | X-ray repair complementing defective repair in Chinese hamster cells 3 | 0.70 | 0.016 |
| ENSGALG00000011621 | insulin like growth factor 2 receptor | 0.66 | 0.018 |
| ENSGALG00000004727 | unc-51 like autophagy activating kinase 2 | 0.61 | 0.022 |
| ENSGALG00000027579 | mitochondrial ribosomal protein S6 | 0.72 | 0.030 |
| ENSGALG00000007710 | ras-like protein family member 11A-like | 0.61 | 0.035 |
| ENSGALG00000014166 | translocator protein (18kDa) | 0.79 | 0.036 |
| ENSGALG00000011153 | lymphocyte antigen 75 | 0.69 | 0.036 |
| ENSGALG00000009689 | paraoxonase 2 | 0.71 | 0.038 |
| ENSGALG00000009248 | heat shock 70kDa protein 12A | 0.77 | 0.046 |
| ENSGALG00000020557 | lysine demethylase 4C | 0.76 | 0.047 |
| ENSGALG00000010537 | cyclin-dependent kinase inhibitor 2C | 0.72 | 0.047 |

Downregulated genes in abdominal adipose tissue at 7 d from N=5 samples per diet group; CO, corn oil; FO, fish oil. Adj. P-values are from T-tests, FO vs. CO. Genes represents those that were annotated in the chicken genome (*Gallus gallus* 5.0)

Table 4.4. Genes upregulated in abdominal adipose tissue of FO vs. CO chicks at 14 d

| ENSEMBL ID | Gene Name | Fold change FO/CO | p. adj. |
|---|---|---|---|
| ENSGALG00000035219 | Albumin | 5.42 | 0.001 |
| ENSGALG00000000044 | WAP, follistatin/kazal, immunoglobulin, kunitz and netrin domain containing 1 | 5.85 | 0.001 |
| ENSGALG00000021040 | hydroxyacylglutathione hydrolase-like | 1.82 | 0.001 |
| ENSGALG00000041926 | hexamethylene bisacetamide inducible 1 | 1.35 | 0.002 |
| ENSGALG00000036805 | spectrin beta, erythrocytic | 1.88 | 0.005 |
| ENSGALG00000006491 | ankyrin repeat domain 1 (cardiac muscle) | 36.26 | 0.006 |
| ENSGALG00000006134 | 4-hydroxy-2-oxoglutarate aldolase 1 | 10.30 | 0.006 |
| ENSGALG00000001926 | heat shock protein family B (small) member 1 | 2.18 | 0.007 |
| ENSGALG00000005996 | fibronectin type III and SPRY domain containing 2 | 2.77 | 0.012 |
| ENSGALG00000003243 | RAD54-like 2 (S. cerevisiae) | 1.50 | 0.025 |
| ENSGALG00000046217 | PIT54 protein | 9.24 | 0.027 |
| ENSGALG00000000104 | cryptochrome 4 | 1.40 | 0.031 |
| ENSGALG00000001264 | plexin A2 | 1.59 | 0.036 |
| ENSGALG00000008905 | hyaluronan binding protein 2 | 15.07 | 0.039 |
| ENSGALG00000001369 | hydroxysteroid (11-beta) dehydrogenase 1b | 2.10 | 0.043 |
| ENSGALG00000009450 | dual specificity phosphatase 10 | 1.47 | 0.043 |
| ENSGALG00000009844 | actin, alpha, cardiac muscle 1 | 20.32 | 0.048 |
| ENSGALG00000015937 | fatty acid binding protein 1 | 5.94 | 0.048 |
| ENSGALG00000001626 | urotensin 2 receptor | 5.30 | 0.048 |
| ENSGALG00000042491 | histone cluster 4 H4 | 2.86 | 0.048 |
| ENSGALG00000007383 | heat shock protein family B (small) member 8 | 2.68 | 0.048 |
| ENSGALG00000004727 | unc-51 like autophagy activating kinase 2 | 1.52 | 0.048 |
| ENSGALG00000007205 | cytochrome c oxidase subunit VIa polypeptide 1 | 1.23 | 0.048 |

Upregulated genes in abdominal adipose tissue at 14 d from N=5 samples per diet group; CO, corn oil; FO, fish oil. Adj. P-values are from T-tests, FO vs. CO. Genes represents those that were annotated in the chicken genome (*Gallus gallus* 5.0)

Table 4.5. Genes downregulated in abdominal adipose tissue of FO vs. CO chicks at 14d

| ENSEMBL ID | Gene Name | Fold change FO/CO | p. adj. |
|---|---|---|---|
| ENSGALG00000042396 | solute carrier family 12 member 7 | 0.72 | 0.001 |
| ENSGALG00000037203 | catenin beta 1 | 0.87 | 0.001 |
| ENSGALG00000011566 | glycophorin C | 0.71 | 0.003 |
| ENSGALG00000030613 | platelet derived growth factor receptor beta | 0.80 | 0.003 |
| ENSGALG00000004230 | lipase C, hepatic type | 0.06 | 0.019 |
| ENSGALG00000016419 | Ankyrin repeat and PH domain 2 | 0.76 | 0.027 |
| ENSGALG00000010949 | glycoprotein nmb | 0.05 | 0.027 |
| ENSGALG00000000529 | LIM domain containing 2 | 0.58 | 0.036 |
| ENSGALG00000034502 | stearoyl-CoA desaturase 5 | 0.66 | 0.043 |
| ENSGALG00000001017 | WD repeat containing, antisense to TP73 | 0.79 | 0.046 |
| ENSGALG00000010435 | RAS guanyl releasing protein 3 (calcium and DAG-regulated) | 0.72 | 0.048 |

Downregulated genes in abdominal adipose tissue at 14 d from N=5 samples per diet group; CO, corn oil; FO, fish oil. Adj. P-values are from T-tests, FO vs. CO. Genes represents those that were annotated in the chicken genome (*Gallus gallus* 5.0)

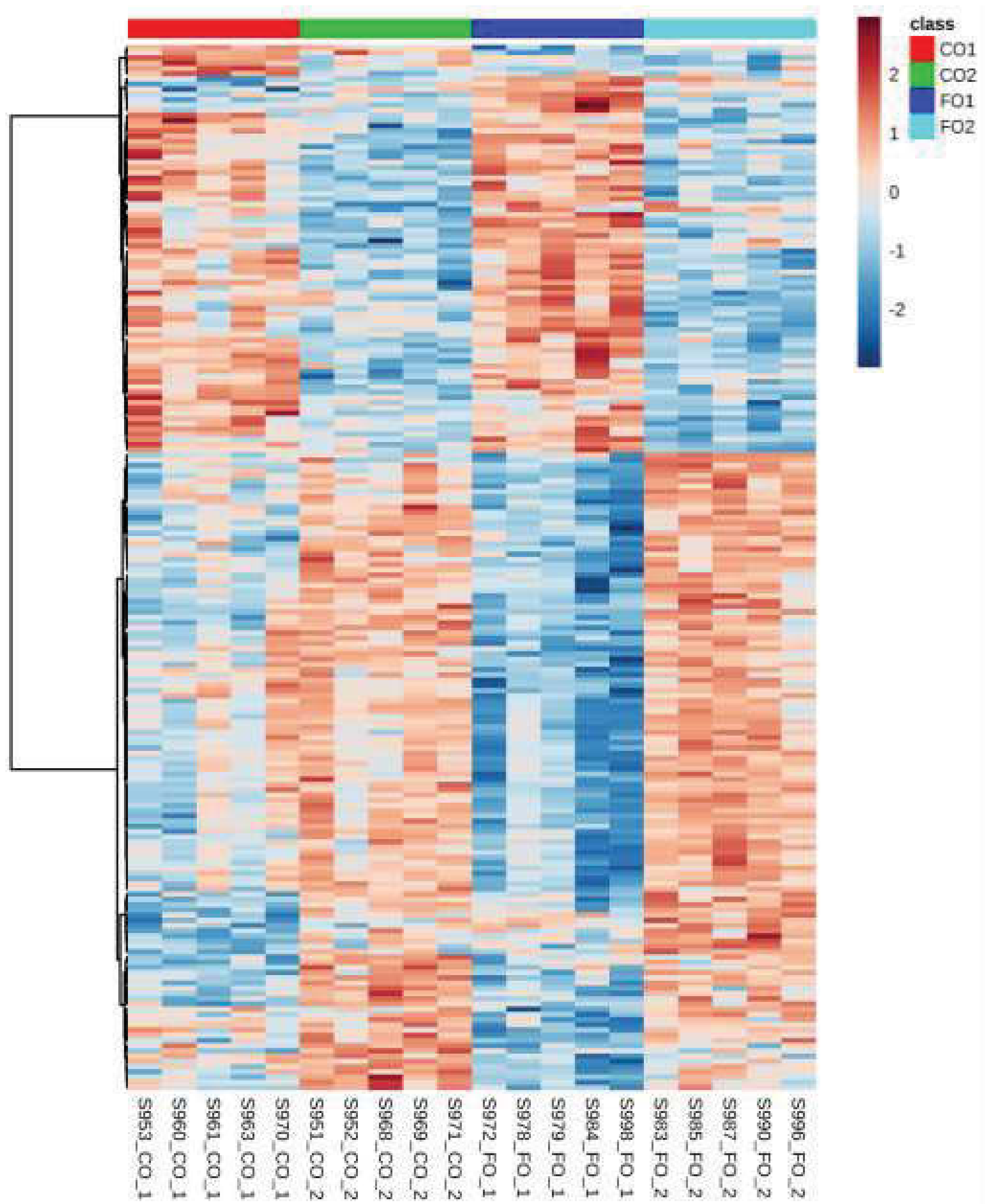


Figure 4.1. Hierarchical clustering separates chicks according to diet and age. The 50 most variable genes between the four groups were analyzed using Pearson's correlation. Distance between samples and genes were determined using complete linkage.

# CHAPTER V.

# TRANSCRIPTOMIC AND METABOLOMIC PROFILING OF CHICKEN ADIPOSE TISSUE: DUAL PURPOSE BENEFIT FOR HUMAN OBESITY AND POULTRY PRODUCTION

A version of this chapter was submitted for publication by: Ronique C. Beckford, Eric D. Tague, Shawn R. Campagna and Brynn H. Voy Transcriptomic and Metabolomic Profiling of Chicken Adipose Tissue: Dual Purpose Benefit for Human Obesity and Poultry Production


### Abstract

Domestic chickens are a valuable yet underutilized set of model organisms for studies relevant to human obesity and adipose metabolism. Chickens and humans share similarities in adipose tissue lipid metabolism, and the in ovo development of the chick enables studies of adipose development that are difficult to perform in other organisms. Fundamental understanding of adipose biology is also important for the poultry industry because broiler (meat-type) chickens are prone to obesity due to consequences of genetic selection for rapid growth. Transcriptomic and metabolomic studies have begun to characterize adipose metabolism in chickens, yielding important insight into mechanisms that control adipose deposition and of leanness. These findings are relevant for both understanding obesity in humans, and for addressing the excess deposition of adipose tissue that plagues commercial broiler production. This review highlights what has been learned about the molecular basis for adipose deposition in chickens using a combination of transcriptomics and metabolomics.



Key words: Adipose tissue, microarray, obesity


### Introduction

Poultry consumption in the United States more than doubled between 1970 and 2007, driven in part by health-conscious consumers seeking a leaner alternative to other meats. Paradoxically, selection of broilers for rapid growth has allowed the industry to meet the increased demand, but has inadvertently increased fat content of the marketable product (Griffin, et al., 1994). For example, the widely used Cobb 500 modern broiler strain was shown to have twice as much relative breast weight but three times as much relative abdominal fat pad weight than Athens

Cobb Random Bred birds, which have been maintained to represent meat-type birds prior to genetic selection (Collins, et al., 2014). Excess fat deposition essentially wastes feed, increasing costs to growers. Beyond the cost of excess feed, carcass fat is an economic liability due to costs associated with processing and with trimming retail cuts to meet consumer preference for a lean protein source. In addition to broilers, excess fat is a concern for the egg-producing and broiler-breeder segments of the poultry industry because it impairs fertility and immune function (Siegel, et al., 2003). Systemic effects of excess fat may also impair skeletal muscle energy metabolism and compromise the efficient growth of lean tissue. Broiler chickens are also a valuable model organism for studies of adipose tissue metabolism and obesity in humans (Voy, et al., 2015). Avian and human (but not rodent or ruminant) lipid synthesis is similar in that liver is the primary lipogenic tissue (Leveille, et al., 1975). Chickens are also naturally hyperglycemic and insulin resistant, comparable to a prediabetes state(Simon, 1989). Finally, heritable susceptibility to obesity in broiler chickens is a polygenic trait, which is reflective of the majority of obesity in humans (Ikeobi, et al., 2002). Therefore, an enhanced understanding of the molecular and cellular pathways that regulate adipose deposition in chickens may yield dual benefit for both production agriculture and for human obesity.

**Adipose tissue development**

Chickens store energy as lipids in both subcutaneous and abdominal adipose depots. Subcutaneous fat is deposited mainly in the femoral and neck region and becomes visible in the embryo at approximately eight to ten days of age. The majority of excess adipose tissue in broilers is contained within the abdominal depot, which begins to develop a few days after hatch and is visually apparent by five to seven days of age. Abdominal adipose deposition proceeds rapidly through increases in both adipocyte number (hyperplasia) and size (hypertrophy). Hyperplasia is

the dominant mechanism of fattening in the first two to three weeks of life, after which hypertrophy increases rapidly and underlies most of the adipose deposition that occurs from four weeks of age to market (Leclercq, 1984). Adipocyte hypertrophy results from uptake and storage of circulating lipids that originate from de novo synthesis in the liver, and from the diet (Leveille, et al., 1975; O'Hea, et al., 1968). Under a typical commercial poultry diet, in which the relatively low amount of dietary fat does not suppress lipogenesis, most stored fatty acids are synthesized in the liver. Circulating VLDL molecules transport fatty acids from the liver to adipose tissue, where they are cleaved by lipoprotein lipase, transported into the adipocyte, re-esterified with glycerol and stored as triglyceride.

While the role of liver in control of fattening is well-established, adipose tissue actively influences its own mass through local and systemic actions that are coupled to metabolism and transcription within adipose tissue (Havel, 2000). Adipose tissue communicates with other peripheral tissues and the brain through release of metabolites, hormones and peptides into the circulation (Trayhurn, et al., 2005). Components of the adipose secretome regulate feed intake, insulin sensitivity, and many other functions that synergize to maintain energy balance (Luo, et al., 2016; Pardo, et al., 2012; Peinado, et al., 2012). Production and release of many of these secreted factors is directly tied to adipocyte metabolism and size through transcriptional mechanisms and metabolic feedback metabolites (Lehr, et al., 2012; Ren, et al., 2012; Schwartz, et al., 1997). Therefore, thorough characterization of the adipose tissue metabolome and transcriptome is critical towards understanding the control of adiposity in any species, including chicken.

**Models of differential fatness**

Much of what is known regarding mechanisms of fattening in chickens has resulted from the study of genetic lines that differ in fatness due to phenotypic selection. Divergent selection is particularly effective in chickens due to the extensive sequence variation and high frequency of recombination within the chicken genome (Wong, et al., 2004). The most dramatic example of the efficiency of selection is the set of high weight (HWS) and low weight (LWS) lines developed by Siegel (Dunnington, et al., 1996). After thirty-seven generations of selection, body weight differed between these two lines by ~ 8-fold, with marked differences in feed efficiency, body composition, egg production, growth hormone, and immune-competence (Siegel, et al., 2003). Divergent selection has been used to produce various experimental lines of chickens that differ in fatness. Groups with significant differences in adiposity have been produced by selection on plasma VLDL level (i.e., hepatic delivery of lipids), plasma glucose level and residual feed intake, as well as directly on abdominal fat mass (Buyse, et al., 1992; Hermier, et al., 1984; Hermier, et al., 1991; Leclercq, et al., 1987). Only a few generations of selection are necessary to yield significant differences in adiposity. For example, relative adipose mass differed by approximately 2-fold by the third generation in the French Fat line (FL) and Lean Line (LL) chickens, which were produced by selection on abdominal adipose mass (Hermier, et al., 1984).

**Omics approaches to understand adipose deposition**

***Transcriptomics:*** Global gene expression analysis has largely moved from microarrays to RNA sequencing (RNAseq). RNAseq enables digital quantification of gene expression through the ability to count transcripts associated with each gene in the genome. Methods for RNAseq have become relatively standard, particularly due to the prevalence of commercial kits for RNA isolation and library construction. Originally, RNA sequencing primarily focused on quantifying

protein-coding mRNA sequences. More recently, the evolving recognition that smaller, non-coding RNAs play important regulatory roles has prompted the use of specific RNA isolation and library preparation methods that allow this population of RNAs to be sequenced and quantified along with longer, coding transcripts. Transcriptome studies often identify hundreds of genes that are differentially expressed, raising the challenge of extracting biological meaning from the data. Pathway discovery is enabled by informatics-based geneset enrichment tools, such as the Database for Annotation, Visualization and Integrated Discovery (DAVID) (Huang, et al., 2008). These tools take as input a set of genes, such as a list of differentially expressed genes, annotate them functionally using Gene Ontology (GO) terms and known pathway (e.g., KEGG) membership, and then identify functions and pathways that are statistically over-represented compared to what would be expected by chance based on comparisons to the species' corresponding genome or to all genes on the array used.

***Metabolomics and lipidomics:*** An inherent assumption of using transcriptomics to understand metabolic physiology is that metabolism can be inferred by querying expression of genes that are involved in energy metabolism. Metabolomics and lipidomics are approaches that directly profile metabolic consequences by globally measuring metabolites themselves, rather than the expression of enzymes that catalyze their production. Conceptually, both metabolomics and lipidomics share the goal of comprehensively profiling the abundance of cellular metabolites. However the disparate chemical properties of molecules comprised in the metabolome and lipidome dictate very different extraction and detection methods, resulting in bifurcation into the two sub-disciplines of metabolomics and lipidomics (Dettmer, et al., 2007) .

Metabolomic studies fall into two broad classes, targeted and untargeted analyses (Patti, et al., 2012). Targeted metabolomics is designed to look specifically for known compounds or

biomarkers of interest. These are often carried out on instruments such as triple quadrupole mass spectrometers where selectivity can be achieved using tandem mass spectrometry wherein each metabolite is detected using very specific parent mass and fragmentation patterns. These experiments, while limited in the overall scope of molecules detected, are very useful for quantification through the use of selected reaction monitoring protocols and internal standards (Lang, et al., 2010; Yerramsetty, et al., 2013). Advances in instrumentation, such as the quadrupole orbitrap (Q-Exactive , Thermo Scientific), will continue to expand the scope of targeted experiments as high resolution mass analyzers enable metabolite identification from untargeted data based on MS/MS fragments (Kouassi Nzoughet, et al., 2017).

Untargeted metabolomics involves analyzing samples in a "shotgun" approach where a broad mass to charge (*m/z*) window is scanned as the sample is analyzed to detect as many molecules as possible. By modifying parameters like extraction solvent, mobile phases, columns, and ionization source it is possible to capture the changes of hundreds of metabolites within a single untargeted analysis (Patti, 2011). The advancement in the sensitivity and mass accuracy of high-resolution mass spectrometers is making untargeted analyses more routine, and it is now becoming feasible to achieve good levels of quantitation during untargeted experiments (Lu, et al., 2010). In addition to identifying chromatographic peaks based on exact masses and retention times, untargeted data can be mined for spectral features that represent metabolites but are not currently annotated in a database. Features that are quantitatively affected by a treatment of interest (e.g., those that differ between fat vs. lean adipose tissue) or that correlate with known metabolites of interest can then be probed for putative chemical composition and structure using orthogonal methods.

Platforms for lipidomics are much more developed than metabolomics because of their more uniform structural properties and composition. Lipidomics methods are subdivided based on head group, water solubility, and/or polarity further requiring different techniques for proper identification (Wenk, 2005). Phospholipids (PL) can be identified by head group and combined fatty acid composition using liquid Chromatography-Mass Spectrometry (LC-MS) because of their aqueous solubility, but the composition of each fatty acid tail and position of double bonds within those fatty acids cannot be discriminated. Gas Chromatography-Mass Spectrometry (GC-MS) can provide details about length and location of unsaturation on each tail of a PL (Castro-Gomez, et al., 2017). Samples must be derivatized to the FAME (Fatty Acid Methyl Ester) before being analyzed (Basconcillo, et al., 2008).

Several compound databases, such as LIPID Metabolites and Pathway Strategy (http://www.lipidmaps.org), the Human Metabolomics Database (http://www.hmdb.ca), and the METLIN Metabolite Database (http://metlin.scripps.edu), serve as catalogs for identification of from mass spectrometric analyses. Bioinformatics resources such as the Kyoto Encyclopedia of Genes and Genomes (http://www.genome.jp/kegg/), and Interactive Pathway Explorer IPath 2.0 (Yamada, et al.), (http://pathways.embl.de/) are useful for mapping compounds in relevant biochemical pathways. Metaboanalyst (www.metaboanalyst.ca), provides end users a web interface to process, normalize, and plot metabolomics data while recent updates have added features integrating pathway analysis for metabolites and genes. The Small Molecule Pathway Database (www.smpdb.ca) uses a visually appealing cellular map to link metabolites, enzymes, and their interactions to one another.

**Insight into control of adipose tissue growth and metabolism in chickens from transcriptomics and metabolomics**

Recent transcriptomic studies of adipose tissue have provided valuable insight into metabolic pathways that contribute to adipose deposition in chickens (Huang, et al., 2015; Ji, et al., 2014; Resnyk, et al., 2015; Zhuo, et al., 2015). A unique feature of these studies when considered collectively is that the genetic backgrounds and underlying basis for differences in fatness differ considerably between studies, creating the ability to robustly associate molecular pathways to fatness. The French FL and LL lines have been intensively studies during the ~ 35 years since their initial selection (Baeza, et al., 2013). These lines were produced from commercial flocks by divergent selection on abdominal fat pad weight (Leclercq, 1984; Leclercq, et al., 1990). The FL birds have approximately 2.5X as much abdominal adipose tissue but lower breast muscle mass than LL birds. Food intake and growth rates are similar between the two lines, but FL birds are hypoglycemic and hypertriglyceridemic compared to LL. Plasma VLDL levels are predictably higher in FL than LL birds, but not until five weeks of age. Adipocyte hypertrophy and hyperplasia appear earlier, and are apparent in FL vs. LL as early as two weeks of age (Hermier, et al., 1989). This set of lines therefore highlights the fact that intrinsic differences in adipose tissue contribute significantly to fattening, in addition to contributions made by liver.

An initial transcriptomic study with these lines was performed using a custom EST microarray (Del-Mar 14K Chicken Integrated Systems microarray (NCBI GEO Platform # GLP1731)), over a range of ages (1, 3, 5, 7, 9 and 11 weeks of age) (Resnyk, et al., 2013). Genes that were differentially expressed between the two lines, regardless of age, were enriched in functions related to lipid metabolism, especially at seven weeks of age and beyond. A follow-on study using the same set of birds employed RNAseq to expand identification of differentially

expressed transcripts at seven weeks (Resnyk, et al., 2015). Pathway analysis highlighted both adipogenesis and fatty acid synthesis as contributors to differences in adiposity. Genes in a diverse set of pathways (e.g., axonal guidance, glucocorticoid signaling, various developmental pathways), in addition to lipid metabolism, differed between the two lines. In particular, a number of genes involved in hemostasis were differentially expressed, with upregulation of genes that promote coagulation and thrombosis in LL birds. The authors proposed that these proteases may regulate fat deposition through processing of peptides and hormones that are secreted by adipocytes. Both fatty acid synthase (*FASN*) and stearoyl-CoA desaturase (*SCD*) were significantly upregulated in adipose tissue of FL vs. LL birds. Fatty acid synthase catalyzes the successive reaction steps of de novo lipogenesis that synthesize palmitate from glucose, while SCD introduces a double bond into palmitate. Increased expression of FASN and SCD in FL adipose tissue suggests that de novo lipogenesis in adipocytes contributes to excess adipose deposition. This possibility conflicts with the conventional view that lipogenic rates in avian adipocytes are too low to impact fat storage (Leveille, et al., 1975). However, its role may be under-estimated, and suggests further study. Collectively, comparison of the transcriptomes of FL and LL highlight that increased lipid storage impacts multiple components of adipose tissue function.

Feed efficiency, the ability to convert feed consumed to body weight, is a critical parameter for efficient broiler production. Birds with low feed efficiency tend to consume more food than birds with high feed efficiency and to preferentially convert feed to adipose tissue rather than breast muscle. Zhuo, et al., (2015) used RNAseq to compare the transcriptomes of birds that differed in feed efficiency and, as a result, abdominal adipose mass. Residual feed conversion was used as a selection trait to identify birds with low (LFE) or high (HFE) feed efficiency. Birds in the LFE group had 55% more abdominal fat than HFE birds at 46 d of age. Consistent with older FL and

LL birds, many genes involved in fatty acid uptake and triglyceride storage were upregulated in LFE birds. Expression of *SCD* was also increased in LFE vs. HFE adipose tissue, although FASN was not described as differentially expressed. Genes in pathways related to cholesterol metabolism (e.g., cholesterol synthesis and transport, steroidogenesis), and in the transcription factors (e.g., sterol regulatory element binding proteins) that regulate expression of cholesterol metabolizing enzymes were also differentially expressed between LFE and HFE. Cholesterol synthesis genes were increased in LFE vs. HFE adipose tissue, suggesting that increased cholesterol storage contributes to lipid droplet expansion and adipocyte hypertrophy in in LFE birds. In general, the pathways that differed between LFE and HFE birds are similar to those differing between FL and LL (Resnyk, et al., 2015), despite the marked differences in selection criteria used to produce these two sets of study groups. This raises the possibility that feed efficiency and adipose deposition are genetically correlated, driven by common molecular pathways. If so, understanding mechanisms that underlie adipose deposition may also improve the understanding of feed efficiency.

Advances in RNAseq technology allow profiling of both coding and small non-coding RNA species. Integration of the two types of data enables the simultaneous identification of differentially expressed regulatory RNAs and their mRNA targets, and to associate these to phenotypes of interest. This strategy has been used to identify miRNAs that regulate expression of lipogenic and adipogenic genes, and potentially fat deposition, in chicken's (Huang, et al., 2015) .Birds with high and low abdominal fat pad weights were selected from an F2 cross of Beijing-You, a slow-growing Chinese breed, and a commercial Cobb-Vantress broiler line. Abdominal adipose tissue was for profiling of both miRNA and mRNA using RNAseq. Differential expression analysis identified 430 protein-coding genes and 62 miRNAs that differed significantly between the two groups of birds. Downstream targets of the miRNAs were predicted and compared to the

set of protein-coding genes, identifying 106 miRNA-mRNA pairs that were coordinately expressed in lean vs. fat birds. Functional enrichment analysis revealed that the coding genes within these pairs were involved in pathways related to both adipocyte hypertrophy (e.g., fatty acid synthesis and metabolism) and adipogenesis (Wnt receptor signaling). In vitro experiments verified the role of select miRNAs in proliferation and differentiation of chicken preadipocytes. This study therefore took RNAseq to the next step by identifying miRNAs that can regulate both adipocyte size and number in chickens. Sequencing and characterization of other classes of non-coding RNAs will almost certainly expand this set of molecular control mechanisms for adipose deposition in chickens. To this point, the repertoire of long con-coding RNAs (lncRNAs) that are expressed in chicken adipose tissue was recently characterized and correlated with co-expressed mRNAs (Muret, et al., 2017).

Despite variation in the origins and methods used to produce fat and lean lines in each of these three studies, each associated upregulation of PPARG with increased adiposity. PPARG encodes a transcription factor that plays a dual role in adipose deposition (Brun, et al., 1997). In preadipocytes, PPARG orchestrates the transcriptional changes that are required for development of a lipid storage phenotype, while in mature adipocytes it is important in maintaining this phenotype. At the ages studied (~ 6-7 weeks of age), hypertrophy is the primary mechanism of fat deposition in broilers. Therefore increased PPARG expression in these models likely signifies its role in mature adipocytes. However, adipocyte number, which reflects PPARG's role in adipogenesis, is significantly increased in FL vs. LL chicks by approximately two weeks of age, well before adipocyte size diverges and prior to increases in plasma VLDL (Baeza, et al., 2013). Other adipogenic proteins (e.g., Kruppel-like transcription factor 15, follistatin-like 1), albeit with less well-defined roles than PPARG, were also differentially expressed in one or more

comparisons of lean vs. fat groups. Upregulation of PPARG may therefore indicate that adipocyte number also plays an important role in fat deposition, even in mature birds.

In contrast to transcriptomics, which was enabled by the development of microarrays in the mid-1990s (Bumgarner, 2013), metabolomics has only recently emerged as tool used to query physiology. Comparisons of the plasma metabolomes of the French FL and LL lines of chickens highlights the insight that can be gained from global metabolite profiles (Baeza, et al., 2015 ). Circulating levels of several amino acids were associated with differences in fatness, highlighting protein metabolism as an under-appreciated pathway that may contribute to adipose deposition. Regression modeling determined that plasma lipids and lipid metabolites (i.e., free fatty acids, total cholesterol and phospholipid, β-hydroxybutyrate) combined with glutamine and methionine levels explained 74% of variability in abdominal fat pad weight. These relationships illustrate the utility of using metabolomics and lipidomics to query the intermediates and end products of cellular metabolism, in addition to profiling expression levels of the genes that produce these metabolites.

Transcriptomics and metabolomics/lipidomics are complementary tools that, when combined, can be more informative than either single platform, particularly for studies of metabolism. Metabolomics data can be used to validate that changes in gene expression have functional consequences on how the tissue allocates energy. They can also prompt further investigation of specific changes in expression of genes that may otherwise be overlooked. Transcriptomic and metabolomic studies of adipose tissue were combined to identify pathways that contribute to increased fatness in broilers compared to naturally lean breeds of chickens (Ji, et al., 2014). Two genetically distinct lean lines (meat-type Fayoumi and egg-type Leghorn) with approximately 50% less fat than broilers were used to increase the robustness of comparisons. Many of the same genes identified in transcriptomic comparisons described above were also

differentially expressed between broilers and the lean lines. For example, pyruvate dehydrogenase kinase 4 (*PDK4*), a kinase that promotes oxidation of fatty acids by phosphorylating and inactivating pyruvate dehydrogenase, was expressed at higher levels in both LL vs, FL chickens and in Fayoumi and Leghorn compared to broilers. Leanness was associated with upregulation of genes that regulate the catabolism and oxidation of fatty acids. Fatty acid oxidation in white adipose tissue has classically been dismissed as playing an important role in fat accumulation because rates are relatively low compared to highly oxidative tissues like skeletal muscle. However, growing evidence in mice and humans indicates that activation of this pathway reduces adipocyte size and inflammation (Kusminski, et al., 2012; Marcelin, et al., 2010). Parallel metabolomic profiling complemented the transcriptomic results by revealing that carnitine and acetylcarnitine, which traffic fatty acids intra-cellularly for oxidation, were significantly more abundant in adipose tissue of both lean lines vs. broilers (Ji, et al., 2014). Recently, this pathway was shown to be rapidly upregulated in response to fasting in young broiler chicks and to be coordinately regulated with genes that mediate both adipogenesis and lipogenesis (Ji, et al., 2012; Torchon, et al., 2017). Convergent transcriptomic and metabolomic evidence for its role leanness underscores the complementarity of these two ‘omics platforms.

Omics studies have the potential to identify control points that can be manipulated by diet or other factors to reduce fatness. Transcriptional and metabolomic profiling have been combined to characterize the homeostatic control of adipose metabolism in young broiler chicks in response to a brief period (5 hrs) of food deprivation (Ji, et al., 2012) Fasting significantly altered the expression of > 1200 genes in abdominal adipose tissue of 21 day-old broiler chicks relative to ad libitum fed controls. Many of the same pathways, e.g., lipogenesis, adipogenesis, cholesterol synthesis, that differed between fat and lean comparisons were also affected by a short term fast

(Figure 1). A number of genes (e.g., carnitine palmitoyl transferase, acyl-CoA oxidase, *PDK4*) that were expressed at higher levels in fasted compared to fed adipose tissue were also upregulated in lean lines, suggesting that pathways altered by fasting overlap with those that contribute to heritable differences in leanness. Metabolomic profiling of adipose tissue highlighted a number of amino acids that were affected by fasting, and several genes involved in protein and amino acid catabolism were also differentially expressed in fasted vs. fed bids. Taken together, this suggests a role for amino acid metabolism that may not have been appreciated if either dataset was generated in isolation. Interestingly this is consistent with differences in plasma metabolomes between FL and LL lines (Baeza, et al., 2015).

**Conclusions and future directions**

In addition to its role as a primary source of lean, affordable dietary protein, the domestic chicken presents novel opportunities as a model organism for studies relevant to human nutrition and obesity. Transcriptomic studies using commercial microarray platforms have provided clues into mechanisms of fat development and expansion in this model. Follow-on studies utilizing the discovery power of RNAseq have the potential to identify an expanded set of transcripts and pathways that are fundamentally important to early adipose tissue development, as well as those that promote obesity or maintain leanness. The power of these studies can be expanded in the future by integrating transcriptomics with tissue metabolomics and lipidomics to identify both molecular pathways and the corresponding effects on adipose metabolism.

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## Appendix

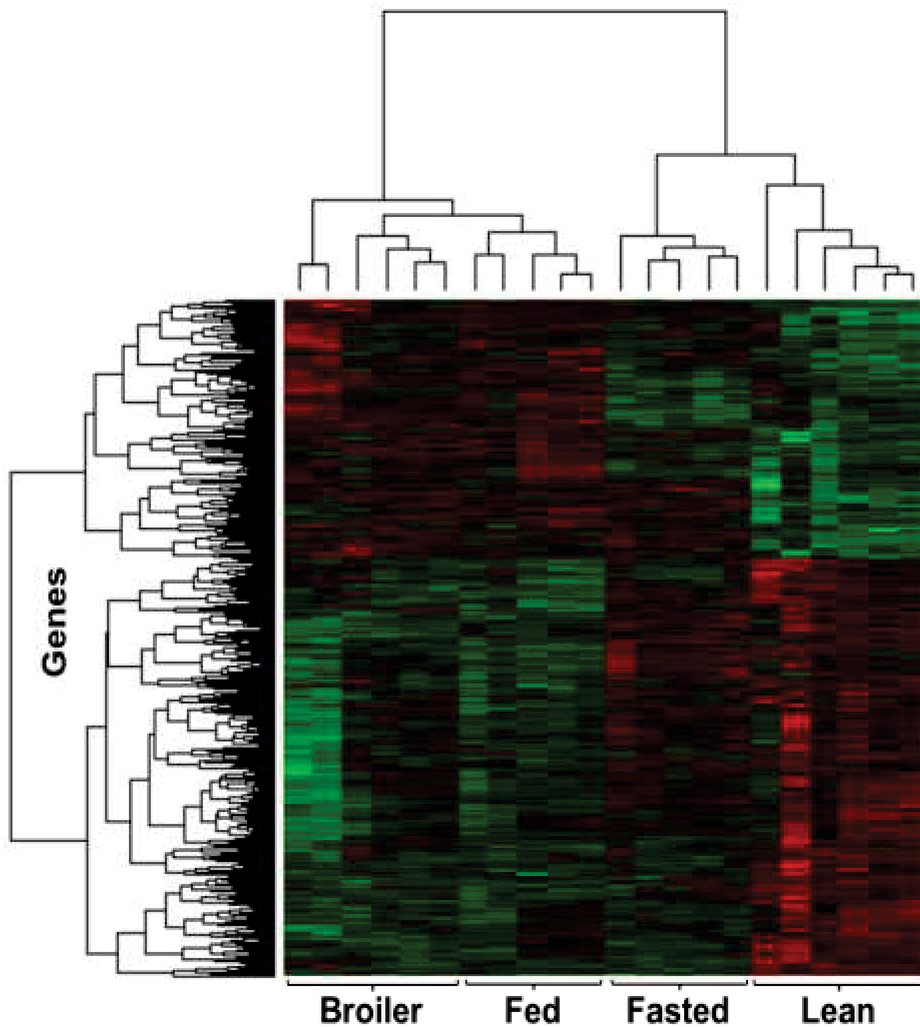


Figure 5.1. Similarities in adipose tissue gene expression profiles between genetically-driven leanness and fasting. Unsupervised clustering was used to visualize similarities in gene expression profiles from abdominal adipose tissue of fed and fasted (five hours) 21 day-old broiler chicks (Ji, et al., 2012), and from fed adult broilers and genetically lean Fayoumi chickens (Ji, et al 2014). Expression profiles from fasted broiler chicks were more similar to those of the genetically distinct, lean Fayoumi line than to profiles from chick or adult broilers that were fed.

## CHAPTER VI.
## CONCLUSION AND FUTURE DIRECTIONS

This study utilized a novel approach to provide the developing embryo with omega 3 polyunsaturated fatty acids. The primary goal was to determine the resultant effect on adipose tissue development and metabolism in the offspring. Feeding broiler breeder hens a diet enriched in long chain polyunsaturated omega 3 fatty acids was sufficient to alter the fatty acid milieu of the yolk consequently influencing the phospholipid profile of the brain, liver, and adipose tissue of the chicks at hatch. Post hatch, chick performance up to two weeks old was not altered. Despite no differences in growth and adiposity, FO chicks had a higher frequency of smaller adipocytes compared to the CO chicks. Though the levels of eicosapentaenoic acid and docosahexaenoic acid decreased significantly at 14 days the impact on adipocyte size and gene expression was still evident. QPCR results found that lipoprotein lipase expression was reduced while the expression of the transcription factor PPARγ and its co-activator PPARγGC1β was increased in the FO chicks. This suggests that there was an increase in adipogenesis and a reduction in uptake of fatty acids in the FO chicks. Profiling the proteome and transcriptome indicated that FO chicks may also have a reduction in gluconeogenesis, lipid metabolism, and lipid trafficking in the adipocyte. Though we anticipated that there would be an impact on fatty acid oxidation, there were no differences in the expression of marker genes in the peroxisome and the mitochondria. Combined, these results suggests that early exposure of the developing embryo to long chain omega 3 polyunsaturated fatty acids through the maternal diet may be a way to address excess fat deposition in the offspring. Though novel, this approach identifies a number of potential mechanisms that would warrant further investigation. Understanding these mechanisms and how they can be regulated could be beneficial to both humans and poultry. From a medical point of view, suppressing fat deposition in humans will reduce the onset of obesity and its associated co-morbidities. On the other hand,

reducing excess fat deposition in chicks would improve feed efficiency and decrease processing cost. Since this study was conducted for two weeks post hatch, it may be necessary to lengthen the duration and combine this method with another feeding regime to sustain its impact over a longer time period. Therefore future studies are needed to determine how much longer this effect would remain and what approach could be used to enhance its effect. For example, fasting may be required to increase utilization. Also if this method was to be translated to humans, identifying the appropriate time of consumption during pregnancy, length of exposure, and the required intake to obtain a substantial impact would be important.

# APPENDIX

## Enriching the starter diet in Omega 3 polyunsaturated fatty acids reduces adipocyte size in broiler chicks

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## Abstract

Epidemiological studies associate perinatal intake of eicosapentaenoic acid (EPA, 20:5, n-3) and docosahexaenoic acid (DHA, 22:6, n-3)) with reduced adiposity in children, suggesting that these fatty acids may alter adipose tissue development. The objective of this study was to determine if enriching the perinatal diet in EPA and DHA reduces fat deposition in juveniles. Cobb 500 broiler chicks were fed isocaloric diets containing fat (8% wt:wt) from fish oil (FO), lard (LA), canola oil (CA), or flaxseed oil (FL) from 7 to 30 d of age. Adiposity (abdominal fat pad wt/body wt) at 30 d was not significantly affected by diet, but FO significantly reduced adipocyte size, increasing the abundance of small adipocytes. Plasma non-esterified fatty acid (NEFA) levels suggest that reduced adipocyte size was due in part to enhanced mobilization of fatty acids from adipose tissue. Our work indicates that dietary EPA and DHA effectively reduce the size of developing adipocytes in juveniles, which may limit adipose deposition and provide metabolic benefits.

Keywords: Childhood obesity, Obesity, Fatty acids, Adipose tissue, Model Organism

## Introduction

Approximately 27% of children in the U.S. are classified as overweight or obese by age five (Cunningham, et al., 2014). Obese children are more likely to be obese adults, and both childhood and adult obesity increase the risk of cardiovascular disease, diabetes and other co-morbidities (Freedman, et al., 2001). Limiting excess fat accumulation in the first few years of life is therefore therapeutically important for children and for prevention of adult obesity. A plethora of studies have identified factors that influence adiposity in mature animals and humans, in which changes in adipocyte size are the primary basis for differences in fatness. Much less is known about control of adipose mass in juveniles, when both adipocyte hypertrophy and hyperplasia actively contribute to fat deposition.

Polyunsaturated fatty acids of the n-3 and n-6 series differentially regulate preadipocyte proliferation, adipogenesis, and triglyceride storage, all of which contribute to deposition of adipose tissue prior to adolescence. Omega-6 PUFAs tend to be pro-adipogenic, while LC n-3 PUFAs (particularly EPA and DHA) attenuate lipid accumulation and promote an oxidative adipocyte phenotype (rev. in (Kopecky, et al., 2009)) Large-scale studies in mother-child pairs have associated n-6 PUFA intake with increased adiposity in children, while an inverse relationship has been demonstrated with dietary n-3 PUFA (Donahue, et al., 2011; Vidakovic, et al., 2016). These associations suggest that the types of fatty acids consumed early in life may influence the course of adipose development and subsequently impact the predisposition for obesity.

Avian models are useful for testing the effects of diet on early adipose development because chicks eat independently at hatch, allowing direct manipulation of the diet very early in life. Broiler chickens in particular are a valuable, polygenic model of susceptibility to obesity due to inadvertent consequences of selection for rapid growth (Cartwright, 1991). Broiler chicks begin

to deposit excess abdominal fat compared to other breeds within two weeks of hatch. In contrast, most rodent models of obesity are monogenic, or are induced by feeding a diet very high in fat. We used Cobb 500 broiler chicks, one of the most widely-used commercial broiler lines, to determine if providing EPA and DHA in the diet early in life attenuates adipose tissue deposition in juveniles. Diets enriched in fish oil (as a source of EPA and DHA) were compared to diets containing equal amounts of lard, canola oil, or flaxseed oil to evaluate the effects relative to other types of fatty acids. Experimental diets were provided beginning at seven days of age to coincide with initial deposition of the abdominal depot, and to focus on a developmental window in which hypertrophy and hyperplasia contribute comparably to adipose growth.

## Materials and Methods

### Animals and diets

All animal procedures were approved by the University of Tennessee Institutional Animal Care and Use Committee. Forty mixed-sex Cobb 500 broiler chicks were fed ad libitum a commercial starter diet from hatch until 7 d, then switched to one of four experimental diets. Experimental diets were produced by adding fat (8% wt:wt) from lard (LA), canola oil (CA), flaxseed oil (FL) or fish oil (FO) into a commercially-formulated starter diet base. Final energy content of each diet was 3018 Kcal/kg. Chicks were fed experimental diets from 7 to 30 d. At 30 d, chicks were weighed, then euthanized by $CO_2$ asphyxiation, and tissues and blood were collected for experimental procedures. Complete experimental details are provided in Supplemental Information.

### Statistical analysis

Data were analyzed for effects of diet using ANOVA, implemented in SAS (version 9.4, SAS Institute Inc., Cary, NC) with $P < 0.05$ as the criterion for statistical significance. A significant

F-test was followed by post-hoc comparisons using Fischer's least significant difference (LSD) to identify pairwise differences between diet groups.

## Results

### Effects on body composition and growth rate

Fatty acid composition of the diet significantly affected final body weight ($P = 0.045$), with FO birds weighing less than those in the LA group (Table 1, $P = 0.02$). Neither absolute nor relative (adjusted for body weight) weights of the abdominal adipose depot or of breast muscle differed significantly between diet groups ($P > 0.05$). Plasma levels of NEFA ($P = 0.002$), but not glucose ($P = 0.130$) were affected by diet, with increased NEFA in FO chicks compared to each of the other diet groups. As expected, the fatty acid profile of the abdominal fat reflected the dietary fatty acid composition (Supplemental Table 1). Feed intake did not differ across diets (data not shown).

Despite similarities in fat pad weight, diet significantly affected abdominal adipocyte volume ($P = 0.020$). Average adipocyte size was smallest in FO chicks, differing significantly from chicks fed LA or CA diets (Table 1). Adipocyte number varied with diet (Table 1), but differences were not statistically significant ($P = 0.093$). Fish oil promoted a shift in adipocyte size, favoring the abundance of relatively small adipocytes compared to diets enriched in LA or CA (Figure 1A). The frequency of very small ($< 2000\ \mu m^3$) adipocytes was significantly increased in FO vs. either LA or CA ($P < 0.05$). Conversely, frequencies of cells in each size bin beyond $4000\ \mu m^3$ were lower ($P < 0.05$) in FO vs. CA, and in FO vs. LA for most bins. Adipocyte volumes tended to be smaller in chicks fed the FL diet, with frequencies intermediate between those of FO and LA or CA in most size bins.

### Effects on Relative mRNA Expression in Visceral White Adipose Tissue and Liver

Dietary fat source significantly influenced expression of PPAR gamma (PPARG), early growth response transcription factor 1 (EGR1), patatin-like phospholipase domain containing

protein 8 (PNPLA8), and pyruvate dehydrogenase kinase 4 (PDK4) in abdominal adipose tissue (Figure 1B). Both PPARG and EGR1 were expressed at significantly lower levels in FO and CA chicks relative to LA. Expression of PNPLA8 was significantly reduced in FO, CA and FL compared to LA. Genes associated with fatty acid oxidation (acyl-coenzyme A oxidase 1 (ACOX1) and carnitine palmitoyl acyltransferase 1 (CPT1)), lipogenesis (fatty acid synthase (FASN)), lipid storage (lipoprotein lipase (LPL)), gluconeogenesis (phosphoenolpyruvate carboxykinase 1 (PCK1)), and inflammation (chemokine C-C ligand 20 (CCL20), colony stimulating factor 1 receptor (CSF1R)) were not significantly affected by dietary fat type. In liver, expression of ACOX1, but not CPT1 or FASN, was significantly affected by diet (Figure 1C). Expression of ACOX1 was higher in FO vs. all other diet groups.

## Discussion

A number of studies in rodents have shown that dietary fish oil can attenuate the obesogenic effects of a high fat diet. These studies have largely used mature animals, in which growth-related adipose expansion has ceased and changes in adipose mass result from effects on adipocyte size. In contrast, we focused on the first few weeks after hatch to capture the period when the abdominal depot develops and rapidly expands through both adipocyte hyperplasia and hypertrophy (Deaton, et al., 1985). Evaluating the effects of fish oil on these pathways is important because both contribute to childhood obesity (Landgraf, et al., 2015; Spalding, et al., 2008). A specific benefit of using an avian model, compared to rodents, is that they lack UCP-1 and thus the capacity for adipose browning. This is valuable because recent studies indicate that at least part of the anti-obesity effects of fish oil in rodents are due to induction of beige adipocytes in white adipose depots (Kim, et al., 2015). Therefore our model enables us to evaluate the specific effects of dietary FO on white adipocytes.

Unlike comparable diet studies in mature chickens e.g., (Gonzalez-Ortiz, et al., 2013), we did not find an effect of dietary n-3 PUFA on adiposity. This may be due to the relatively brief period of feeding (23 days), or because fatty acid type was not sufficient to influence the inherent stimulus for rapid adipose deposition during this age window. However, diets that were enriched in n-3 PUFA (particularly FO) favored the abundance of small adipocytes relative to diets enriched in LA or CA. Although adipocyte hypertrophy is a normal component of adipose development, excessive hypertrophy presents very early in obese children and promotes insulin resistance and adipose inflammation (Landgraf, et al., 2015). Therefore, the ability of dietary n-3 PUFA to reduce adipocyte size in juveniles, even in the absence of decreased fat mass, may provide metabolic benefits for children prone to obesity.

Adipocyte size results from a balance between fatty acid uptake and mobilization, particularly in species (e.g., avians and humans) in which de novo lipogenesis in adipose tissue is relatively modest. Increased plasma NEFA levels suggest that enhanced fatty acid mobilization may have contributed to reduced adipocyte size in FO chicks. Interestingly, elevated NEFA, reduced adipocyte size, and enrichment of adipose tissue in EPA and DHA are also found in genetically lean lines of chickens relative to obesity-prone broilers (Ji, et al., 2014). Both DHA and EPA have been shown to stimulate lipolysis and reduce adipocyte lipid accumulation in vitro (Barber, et al., 2013; Kim, et al., 2006). Consumption of FO may therefore have reduced adipocyte size by promoting mobilization of fatty acids that are then oxidized by other tissues. Hepatic expression of ACOX1, the rate-limiting enzyme for oxidation of very long chain fatty acids in peroxisomes, was increased by dietary FO. However, expression of CPT1, which regulates mitochondrial fatty acid oxidation and is often co-regulated with ACOX1, was not significantly affected by diet in liver. Therefore increased expression of ACOX1 in the FO group may reflect a

specific response to the abundance of very long chain PUFA (EPA and DHA), rather than a net increase in hepatic fatty acid catabolism. Dietary EPA has also been shown to enhance fatty acid oxidation within white adipocytes (LeMieux, et al., 2015). However, our data do not indicate that this pathway contributed to reduced adipocyte size in FO and FL chicks, based on expression of CPT1 and ACOX1. It is also possible that FO, and to some extent FL, increased the abundance of smaller adipocytes by suppressing their progression through differentiation, rather than altering the balance between lipid storage and mobilization. This possibility is supported by a study in which dietary perilla oil (~ 52% ALA) down-regulated the later stages of adipocyte differentiation in rats (Okuno, et al., 1997). Additional characterization with stage-specific markers of adipocyte differentiation is necessary to investigate this possibility in our study.

None of the genes that we profiled were specifically affected by the diets (FO and FL) that reduced adipocyte size. However, all three diets enriched in unsaturated fatty acids reduced expression of PPARG, EGR1 and PNPLA8 compared to LA. PPARG is a well-characterized transcriptional regulator of both adipocyte differentiation and maintenance of the mature adipocyte phenotype (Chawla, et al., 1994). Increased PPARG expression with dietary SFA vs. PUFA-enriched diets is consistent with comparable studies in mature broilers (Royan, et al., 2011). EGR1 is a pleiotropic transcription factor that has been linked to multiple aspects of adipocyte function (Singh, et al., 2015). Elevated EGR1 expression in adipose tissue is associated with obesity in humans and mice, while loss of EGR1 enhances adipocyte metabolism and confers protection from obesity (Zhang, et al., 2013). Decreased expression of this gene in the FL, FO and CA groups relative to LA may therefore reflect beneficial effects of dietary fat quality on adipocyte metabolism. Calcium-independent phospholipase $A_2\gamma$ (iPL$A_2\gamma$, encoded by PNPLA8), is a phospholipase that catalyzes the release of fatty acid side chains from mitochondrial phospholipids

to generate production of eicosanoids and other lipid second messengers that regulate cellular energetics . The specific roles of $iPLA_2\gamma$ in adipose tissue are not known, but $PNPLA8^{-/-}$ mice are resistant to diet-induced obesity, with reduced adipocyte size relative to wild type controls (Mancuso, et al., 2010). Although expression was not consistently associated with adipocyte size in our study, reduced levels across all PUFA-enriched diets suggest that dietary fatty acids may regulate mitochondrial lipid mediators and subsequently adipocyte metabolism through PNPLA8.

In conclusion, we have demonstrated that dietary FO attenuates adipocyte hypertrophy in juveniles that are prone to rapid fat accumulation. The mechanisms underlying this effect remain to be determined, but may include increased mobilization of stored fatty acids for oxidation by other tissues or disruption of adipocyte maturation. Although the effect of diet on adipocyte size did not manifest as significantly less adipose mass during the relatively brief period of feeding used herein, it may nonetheless be sufficient to elicit favorable metabolic effects in children who are prone to obesity.

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**Appendix**

Table 1. Effects of dietary LA, CA, FL and FO on body, adipose and muscle weights and on serum metabolites in broiler chicks[1]

| | LA | CA | FL | FO | P-value[2] |
|---|---|---|---|---|---|
| Body (g) | 1752 ± 46.7[b] | 1650 ± 54.6[ab] | 1694 ± 45.1[ab] | 1562 ± 45.0[a] | 0.045 |
| Breast (g) | 337.9 ± 13.8 | 360.2 ± 16.5 | 354.6 ± 13.6 | 346.1 ± 7.9 | 0.573 |
| Breast (%)[3] | 19.4 ± 1.0 | 21.9 ± 0.6 | 21.0 ± 1.0 | 22.1 ± 1.7 | 0.400 |
| Adipose (g) | 26.0 ± 1.9 | 24.5 ± 1.2 | 22.3 ± 1.2 | 24.3 ± 1.8 | 0.455 |
| Adipose (%)[4] | 1.48 ± 0.09 | 1.51 ± 0.07 | 1.33 ± 0.07 | 1.56 ± 0.12 | 0.499 |
| NEFA (mM) | 6.25 ± 1.98 [b] | 6.49 ± 0.79 [b] | 6.55 ± 1.41[b] | 10.04 ± 0.96[a] | 0.002 |
| Glucose (mg/dL) | 175.7 ± 12.8 | 184.5 ± 10.8 | 189.4 ± 7.0 | 168.1 ± 5.7 | 0.130 |
| Adipocyte volume | 3651 ± 420.7[a] | 3706 ± 184.1[a] | 3098 ± 95.2[ab] | 2546 ±153.1[b] | 0.020 |
| Adipocyte number[5] | 75.4 ± 13.3 | 70.8 ± 9.5 | 93.7 ± 9.8 | 90.3 ±8.1 | 0.093 |

[1]LA= Lard; CA=Canola oil; FL=Flax; FO=Fish oil. Data are means ± SEM for all chicks in each diet group, N=10/group. Means with shared superscripts do not differ significantly; $p<0.05$.

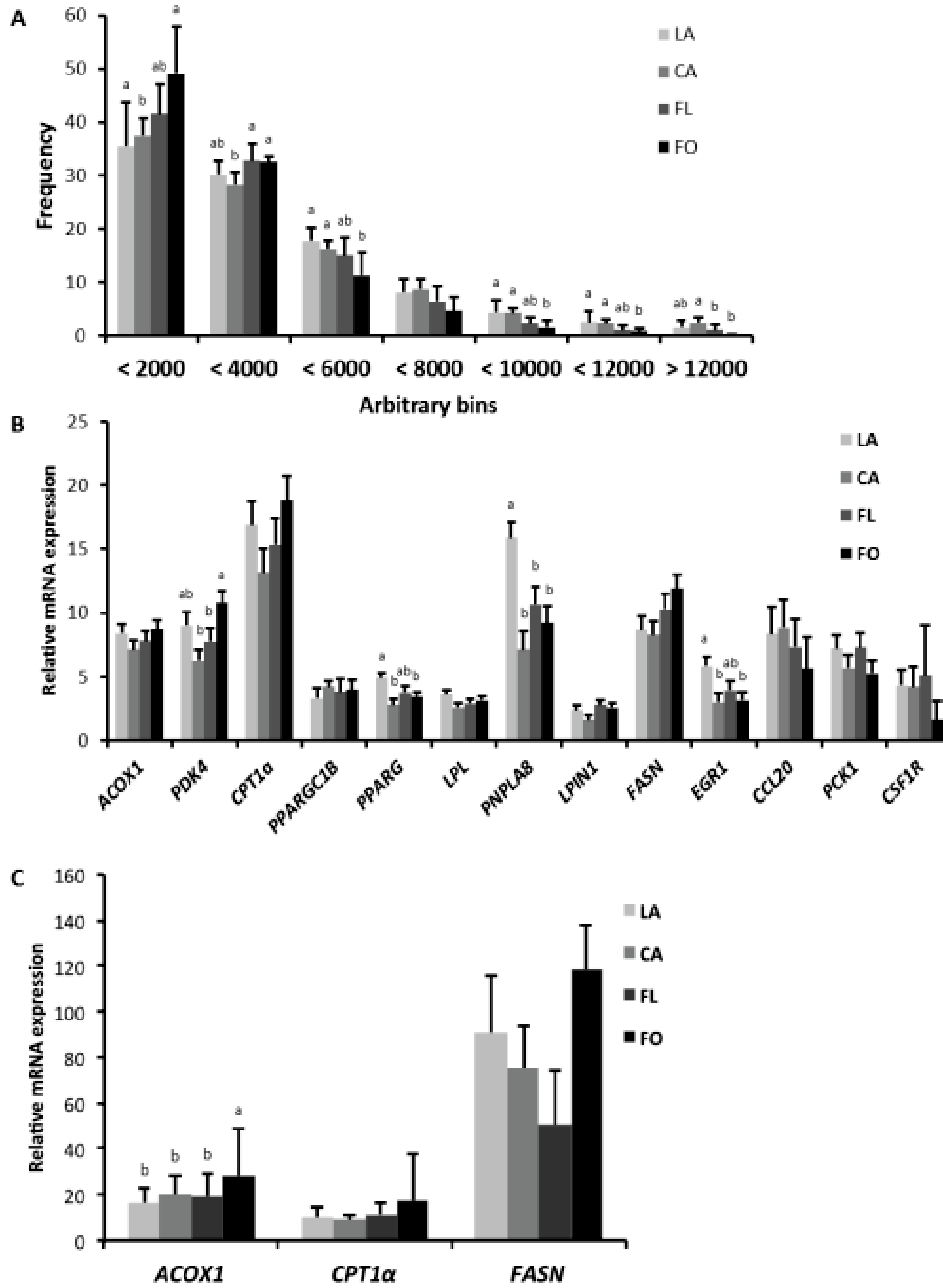
[2] P-value from single factor ANOVA for effect of diet

[3] (Breast wt (g)/body wt (g))*100

[4] (Abdominal adipose depot wt (g)/body wt (g))*100

[5] Calculated from adipocyte volume and adipose depot weight

Figure 1. Effects of dietary FO, FL, CA and LA on abdominal adipocyte size distribution and on gene expression in liver and adipose tissue. (A), Adipocyte area was measured from images of H&E-stained sections of abdominal adipose tissue using Image J (Version 1.48, National Institutes of Health); areas < 500 $\mu m^2$ were removed from analyses. Adipocyte volume ($\mu m^3$) was calculated from area ($\mu m^2$). Frequency distributions were produced by grouping adipocytes into bins based on volume and counting the frequency of cells within each bin. ANOVA was used to evaluate the effect of diet within each bin. Values with different superscripts differ significantly ($P < 0.05$) based on post-hoc testing when ANOVA indicated a significant effect of diet ($P < 0.05$); (B, C). Relative mRNA expression of genes involved in lipid metabolism, adipogenesis and inflammation in abdominal adipose tissue (B), and in lipid metabolism in liver (C) , N=6 birds / diet. Values are group means ± group std. dev.; values with different superscripts differ significantly ($P < 0.05$) based on post-hoc testing when ANOVA indicated a significant effect of diet ($P < 0.05$); Lard (LA), Canola Oil (CA), Flaxseed oil (FL), Fish oil (FO).


A
Frequency
Arbitrary bins
< 2000
< 4000
< 6000
< 8000
< 10000
< 12000
> 12000
LA
CA
FL
FO
B
Relative mRNA expression
ACOX1
PDK4
CPT1α
PPARGC1B
PPARG
LPL
PNPLA8
LPIN1
FASN
EGR1
CCL20
PCK1
CSF1R
C
ACOX1
CPT1α
FASN

**Supplemental Methods**

***Animals and experimental diets:*** Animal husbandry procedures were reviewed and approved by the Institutional Animal Care and Use Committee of the University of Tennessee. Forty 1-d old mixed sex Cobb 500 broiler chicks were utilized in the study. Birds were raised in stacked wire cages under standard management conditions, and had ad libitum access to water and feed. For the first six days, birds were fed a standard commercial broiler starter diet. On day seven, birds were assigned to one of four diets that differed in the type of added fat. Diets were prepared by adding lard (LA) (LA; Refined Lard, Lundy's, USA), canola oil (CA; Pure Wesson 100% Natural, ConAgra Foods Inc., USA), flaxseed oil (FL), and fish oil (FO) (JEDWARDS International Inc., Quincy Massachusetts) at 8% (wt:wt) to a standard broiler starter base diet formulated primarily from corn and soybean (Table 1). The base diet was mixed in one large batch, and the four sources of fat were added to the same base diet. Final energy content of each diet was 3018 Kcal/kg. Experimental diets (those with added oil) were prepared every five days and stored at $4^{0}$C.to minimize oxidation. Chicks were maintained on the experimental diets for 23 days (until 30 d of age). Body weight and feed intake were monitored weekly.

Chicks were euthanized by $CO_2$ asphyxiation. At the time of euthanasia blood was collected by cardiac venipuncture and transferred to 10 ml SST tubes (Fisher Scientific, Pittsburgh, PA). Serum was separated by centrifugation and frozen at -80$^{o}$ C until analyses of circulating metabolites. Abdominal and femoral (subcutaneous) adipose depots were dissected and weighed as indices of adiposity. Breast (pectoralis major) muscle was dissected and weighed to assess muscle deposition. Samples of adipose, breast and liver tissue were subsequently snap-frozen in liquid nitrogen and stored at -80$^{o}$C. Samples of abdominal adipose tissue were also fixed for 24 h at 4$^{o}$C in paraformaldehyde (4%) for determination of adipocyte size by histology. Abdominal fat

was removed and submerged in chilled 4% paraformaldehyde in 0.1 molar sodium phosphate buffer at pH = 7.4 for tissue fixation. Tubes were incubated at 4°C for 12 hour, washed in sodium phosphate buffer at 0.1 molar, and then transferred into chilled sodium phosphate buffer for storage.

***Serum metabolites:*** Commercially available colorimetric assay kits were used to measure serum glucose (Cayman Chemical, Ann Arbor, MI) and non-esterified fatty acid (NEFA) levels (Wako Chemicals, Neuss, Germany).

***Fatty acid analysis:*** Abdominal fat from one randomly selected bird for each treatment was analyzed for phospholipids (PL), neutral lipids (NL), and total lipids (TL) using gas chromatography. Fatty and acids were extracted using a modified Folch method (Ref). Briefly, lipids were separated using thin layer chromatography (TLC) plates, pre-coated with silica gel 60 (Merck, Darmstadt, Germany). Boron tri-fluoride in methanol and heat were used to saponify lipids into fatty acid methyl esters (FAME). Hexane was used to extract and dissolve the FAME. Fatty acid methyl esters were separated 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.

***Adipose tissue histology:*** Abdominal fat samples from three birds in each diet group were embedded, sectioned and stained with hematoxylin and eosin (H&E; two slides/bird) for determination of adipocyte size, as previously described by (Ji, et al., 2014). Briefly, images of three independent fields were captured on each slide under 20x magnification with the Advanced Microscopy Group EVOS XL Core microscope (Fisher Scientific, Pittsburgh, PA). For

consistency, the same person performed all measurements. Image J (Version 1.48, National Institutes of Health) was used to determine adipocyte area, ($\mu m^2$), using microscope settings of 2.8 μm/pixel, and using the restriction that measurements must exceed 500 $\mu m^2$. Frequency distributions were produced by grouping adipocytes into bins based on area and counting the frequency of cells within each bin. Adipocyte number was estimated based on average adipocyte volume and on weight of the adipose depot.

***Real time PCR assay:*** Total RNA was isolated from approximately 200 mg of abdominal adipose tissue and liver from five chicks in each treatment using Invitrogen™ TRIzol™ (Invitrogen, Carlsbad, CA). CDNA was synthesized from 500 ng total RNA in 20 μl reactions using iScript cDNA Synthesis kit (Bio-Rad Laboratories, Hercules, CA). Predesigned and validated primers for quantitative real-time PCR (QPCR) were purchased from Qiagen (Quantitect; Germantown, MD). QPCR was performed in triplicate for each sample using iQ SYBR Green Master Mix (Bio-Rad Laboratories, Hercules, CA), as previously described (Ji, et al., 2014). Expression levels of genes of interest were normalized to expression of TBC1 domain family, member 8 (*TBC1D8*) used as a housekeeper.

Table S.1. Base experimental diet composition

| Ingredient | Amount (%,)[1] |
|---|---|
| Corn | 56.76 |
| Soybean meal | 31.94 |
| Vitamin Premix[2] | 0.05 |
| Choline | 0.20 |
| DL Met | 0.10 |
| Salt | 0.30 |
| Limestone | 1.1 |
| Dicalcium phosphate | 1.4 |
| Fat[3] | 8.0 |

[1] % in diet, wt:wt

[2]Vitamin/mineral premix mix: vitamin A, 30,800 IU; Vitamin $D_3$, 9,250 IU; vitamin E, 153.9 IU; vitamin $B_{12}$, 0.154 mg; riboflavin, 46.2 mg; niacin, 185 mg; pantothenic acid, 84 mg; menadione sodium bisulfite, 16.2 mg; folic acid, 12.3 mg; pyridoxine HCl, 46.2 mg; thiamine HCl, 20.5 mg; biotin, 9.3 mg; choline, 2,944 mg; niacin, 185 mg Cu, 55 mg; I, 7.3 mg; Fe, 366 mg; Mn, 310 mg; Zn, 321 mg; K, 2.23 g; Mg, 1.09 g; Se, 0.48 mg

[3]Supplied as either lard, canola oil, flaxseed oil or fish oil

Table S.2. Effects of dietary LA, CA, FL and FO on fatty acid profiles of phospholipids and total lipids in abdominal adipose tissue[1]

| | Phospholipids lipids (mole%) | | | | Neutral lipids (mole%) | | | |
|---|---|---|---|---|---|---|---|---|
| | LA | CA | FL | FO | LA | CA | FL | FO |
| C14:0 | 2.46 | 1.19 | 1.26 | 19.30 | - | - | - | 8.45 |
| C16:0 | 38.26 | 19.05 | 13.10 | 8.64 | 26.36 | 19.47 | 18.87 | 26.49 |
| C16:1n-7 | - | - | - | - | 7.49 | 4.51 | 5.62 | 12.64 |
| C18:0 | 7.13 | 5.25 | 3.48 | 17.06 | 4.75 | 4.70 | 4.40 | 9.05 |
| C18:1n-9 | - | 41.30 | 18.27 | - | 39.63 | 48.05 | 28.00 | 33.75 |
| C18:2n-6 | 30.24 | 20.45 | 12.35 | - | 4.43 | 18.83 | 16.51 | 14.48 |
| C18:3n-3 | 2.15 | 4.27 | 21.12 | 3.97 | - | 4.44 | 26.59 | - |
| C20:4n-6 | 0.48 | 1.82 | - | 2.08 | - | - | - | - |
| C20:5n-3 | - | - | - | 28.69 | - | - | - | 13.44 |
| C22:6n-3 | - | - | - | 16.68 | - | - | - | 8.18 |

[1]LA= Lard; CA=Canola oil; FL=Flax; FO=Fish oil; N=1/diet

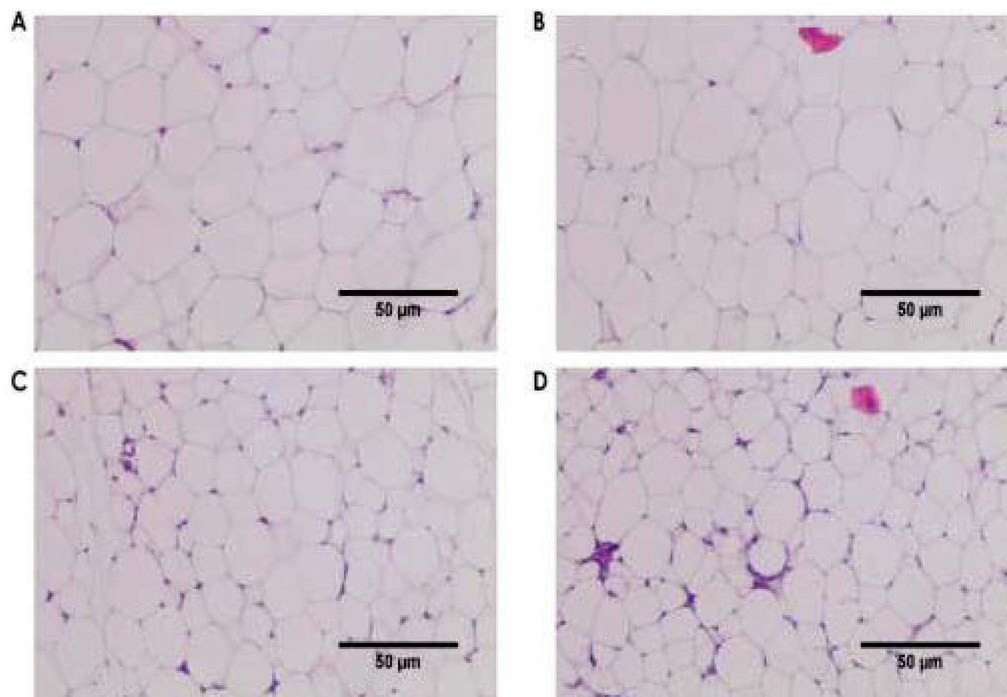



Figure S.1. Representative H&E-stained images of abdominal adipocytes; (A) Lard (LA), (B) Canola Oil (CA), (C) Flaxseed oil (FL), (D) Fish oil (FO); 20X magnification.

# VITA

Ronique C. Beckford was born and raised in Mc Field District Westmoreland Jamaica. After completing his Associate of Science degree at the College of Agriculture Science and Education he migrated to the United States. He attended Tuskegee University in Alabama where he earned a Bachelor and Masters of Science degree in 2010 and 2012 respectively. After working as a research Associate at Tuskegee for 2 years, he moved to the University of Tennessee to pursue a terminal degree. His Ph.D. in Animal Science was conferred in December 2017.