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Broiler chicken development: from genetic regulation to rural Rwandan production

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To the Graduate Council:

I am submitting herewith a thesis written by Robert Ivan Mihelic entitled "Broiler chicken development: from genetic regulation to rural Rwandan production." I have examined the final electronic copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Animal Science.

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Broiler chicken development: from genetic regulation to rural Rwandan production

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ABSTRACT

As the global population continues to increase increasing protein efficiency and sustainability is important to ensure proper nutrient and food security for future generations. Two studies investigating the efficiency of broiler chicken production globally are presented. Reducing the amount of excess fat in broilers could increase efficiency by increasing feed conversion rates. Early exposure to the fatty acids eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) has been shown to reduce fat deposition later in life. Study one investigates the developmental regulation of elongases and desaturases in young and embryonic broiler chickens to determine the tissue and age broiler chicks could efficiently produce EPA and DHA. Targeted RNA sequencing was used to examine expression profiles of subcutaneous adipose tissue -8, -6, -4, +7, and +14 days from hatch. Liver, abdominal, and crop fat were examined at +7 and +14 days. To ensure sustainability globally study two examines methods to increase charcoal efficiency for brooding broilers for small holder farmers in rural Rwanda. Four methods which reflected rising heat back to the chicks were tested. Charcoal use, growth rate, and mortality were measured. Study one found an expression change at +7 day, likely due to dietary changes in fat consumption, alters the pathway broilers could be using to produce EPA and DHA. Even a slight reduction in fat can result in substantial savings for the poultry industry domestically. In Rwanda half house brooding reduced charcoal consumption by >50% leading to greater economic and environmental sustainability.

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CHAPTER 1 INTRODUCTION

Review of literature

Broiler chickens – The most highly consumed source of animal protein

Chicken meat is the most consumed protein source in America with 90% of the population consuming chicken at least once every ~ 2 days (National-Chicken-Council, 2018). The average person in the United States consumed 92 lbs of chicken in 2017 leading the nation to spend a total of $95 billion on chicken meat (National-Chicken-Council, 2018). The United States is the world's largest broiler chicken producer with three of the top five highest producing states, Georgia, Alabama, and North Carolina, located in the Southeast. America exported 16.5% (6.79 billion pounds) of total broiler production in 2017. The largest importers of American chicken are Mexico, Canada, and Hong Kong (National-Chicken-Council, 2018)

In the last 100 years the broiler industry has grown from almost non-existent to one of the most successful agricultural industries. In 1926 Mrs. Wilmer Steele started the first broiler operation with a 500 chicken flock. Her flock was so profitable just 3 years later she built a 10,000 bird house (National-Chicken-Council, 2018). The industry slowly grew until 1948 when USDA and the Great Atlantic & Pacific Tea Company, America's largest grocery store chain at the time, co-sponsored the 'Chicken of Tomorrow' campaign which selected broilers based on carcass characteristics. The goal of the competition was to create a meat type chicken to grow faster and more efficiently than non-selected breeds. The winning birds became the progenitors for modern broilers. Since the 'Chicken of Tomorrow' campaign, producers have continued to select for rapid growth and shorter grow-out times decreasing time till harvest from 16 weeks in 1948 to 44 days today (Coe, 2014). The poultry industry has continued to grow since the 1950's and now directly provides 280,000 jobs and indirectly provides 1.3 million jobs (National-Chicken-Council, 2018).

Excess fat accretion: a consequence of meeting the increased demand for chicken

Inadvertently, as broilers have been selected for rapid muscle growth, there has been co-selection for abdominal fat deposition. Today's broilers have twice the abdominal fat as chickens from 1957 (Havenstein, Ferket, & Qureshi, 2003). Fat deposition is a problem for the poultry industry because it has to be trimmed off at harvest and it reduces the available nutrients for muscle production (Beckford et al., 2017). Poultry feed constitutes approximately 70% the cost of rearing chickens. Nutrients used to synthesize fat instead of muscle are considered a waste as broilers are raised primarily for muscle production. Producers want to maximize meat from the muscle because it is the primary marketable product from broiler chickens.

Human obesity – excess fat with different consequences

Obesity has been a growing epidemic in America since the 1960's with now greater than 1/3 of adults suffering with obesity. From 1962 to 2008 the rate of overweight adult Americans rose from 23% to 62.8% (CDC, 2018a). Overweight and obese are defined as abnormal or excessive fat accumulation that may impair health (WHO, 2018). Body Mass Index (BMI), the measurement of a person's weight divided by height squared, is commonly used to identify overweight and obese individuals. In adults, obesity is characterized by a BMI equal to or greater than 30.0kg/m2,¬ and overweight is a BMI greater than or equal to 25.0kg/m2 (WHO, 2018). Obesity is a major public health issue because it leads to various health concerns including heart disease, stroke, type 2 diabetes, and cancer (Visscher & Seidell, 2001). These detrimental health issues contribute $150 billion to America's yearly healthcare costs. The incidence of obesity begins early in life. Since the 1970's, rates of childhood obesity have tripled among children between the ages of 2-19. One in 6 children in America is now classified as obese (CDC, 2018b). Over weight and obese children are five times more likely to be obese as adults, highlighting the need for early intervention in children (CDC, 2018c). The basis for the increase in childhood obesity is poorly understood and likely includes many factors. Evidence suggest that susceptibility may actually begin before birth through a source described as developmental programming. Developmental programming refers to the fact that dietary and environmental factors during embryonic development can result in long-lasting phenotypic impacts. Numerous studies in humans and other species have shown that adipose tissue is particularly sensitive to the influence of developmental programming. For example, smoking, malnutrition, high fat diets, and exposure to chemicals like bisphenol A have been shown to increase offspring adipose deposition in various species (Veiga-Lopez et al., 2015).

Broilers as a dual purpose-dual benefit model

Broiler chickens are a good model to study adipose development because it benefits both the broiler industry as well as human health. Broiler chickens rapidly develop fat after hatching and are two times fatter than non-selected chickens after just 45 days (Havenstein et al., 2003). Also, the yolk, similar to breast milk, is a direct reflection of maternal fatty acid consumption allowing easy dietary fatty acid manipulation for developing offspring. Manipulations of maternal fatty acid consumption allows for embryonic exposure to varying levels of fatty acids to evaluate their impact on development.

Adipose tissue – fundamentals of growth and expansion

Adipose tissue, commonly known as fat, is composed of adipocytes whose primary functions are energy storage and, in some species, as insulation. Fat is usually located in the abdomen or subcutaneously, but is also found around organs acting as cushion. Adipose tissue consists primarily of mature adipocytes, which comprise ~ 90% of the tissue's weight. A mature adipocyte is

characterized by a single, large droplet of intracellular triglyceride, which acts as an energy reservoir for the body. Functionally, mature adipocytes are characterized by their ability to synthesize, store and mobilize fatty acids for use by other tissues in the body and by adipocytes themselves. Adipocyte formation begins in the embryo, when mesenchymal stem cells first commit to preadipocytes, then terminally differentiate into adipocytes. Before commitment mesenchymal stem cells have the ability to differentiate into adipocytes, myocytes, or osteoblasts. Determination of mesenchymal stem cells is primarily controlled by WNT signaling pathways. The Wnt5a and Wnt10b pathways inhibit adipocyte formation, while Wnt5b promotes adipogenesis. Committed pre-adipocytes differentiate to mature adipocytes through a signaling cascade that is predominantly controlled by the transcription factor peroxisome proliferator activated receptor gamma (PPARγ). This transcription factor, along with other binding proteins, controls the expression of genes that are necessary to confer the characteristics of a mature adipocyte. Adipose tissue growth occurs by either hyperplasia, an increase in the number of adipocytes, and/or hypertrophy, an increase in adipocyte size. Hyperplasia can result from increased proliferation of committed preadipocytes, or from an increase in their differentiation to mature adipocytes. Hypertrophy results from a net increase in the amount of triacylglycerol stored in an adipocyte, which results from a balance between storage and mobilization of fatty acids. Hyperplasia is the predominant method of growth early in development (Jo et al., 2009). In chickens hyperplasia primarily occurs in preadipocytes before differentiation. Hypertrophy of adipocytes occurs during times of excess energy and is most often associated with growth of mature adipocytes in chickens (Figure 1.1) (Beckford et al., 2017; Hirsch & Batchelor, 1976). Stored lipids can be mobilized when membrane bound hormone receptors detect low insulin or high glucagon levels. Insulin is released into the bloodstream by the pancreas in response to high blood glucose. Insulin binds to receptor proteins on muscle, liver, and fat cells activating a molecular cascade which allows glucose to be absorbed into cells. Once in the cell, glucose can either be metabolized, stored as glycogen in the liver, or stored as fatty acids in adipocytes. Glucagon acts in conjunction with insulin to maintain glucose homeostasis by releasing glucose and fatty acids into circulation when blood glucose and insulin are low. Glucagon decreases fatty acid synthesis by promoting lipolysis and release of fatty acids in adipocytes. In response to increasing glucagon levels, hormone sensitive lipase catalyzes intracellular breakdown of stored triglyceride in adipocytes.

In order for stored energy to be obtained fatty acids must first be broken down through the process of β-oxidation. In adipose tissue free fatty acids are released into the blood stream when lipase breaks the bond between glycerol and the fatty acid.

Types of fatty acids

In the early 1900's the view of fatty acids changed dramatically. Due to their high energy density of 9 kcal/mol, it was first hypothesized fatty acids only biological importance was as an energy source. The flawed view was reversed in 1929 by George and Mildred Burr, a husband and wife duo who fed rats fat free diets and reported an increase in deficiency related diseases. They surmised fatty acids, specifically linoleic and its n-3 counterpart α-linolenic acids, were needed for proper biological function and to prevent diseases. George and Mildred Burr's discoveries and the consequential paradigm shift in the view of fatty acids was momentous for future fatty acid research (Spector & Kim, 2015).

Nomenclature of fatty acids has evolved through the years. The most internationally recognized scientific system was established in the Compendium of Chemical Terminology by the International Union of Pure and Applied Chemistry. The nomenclature formula is C:Dn-x where C is the number of carbons in the chain, D is the number of double bonds in the carbon chain, n-x is the location of the fist double bond starting from the methyl end of the carbon chain. Docosahexaenoic acid (DHA; 22:6n-3), for example has a 22 carbon chain with 6 double bonds. The first of the double bonds is three carbons from the methylated end of the carbon chain. This nomenclature method lacks representation of all double bonds in the carbon chain. In an alternate nomenclature which represents all double bonds, DHA is written as 22:6Δ4,7,10,13,16,19 where double bonds are listed starting at the carboxyl end of the chain (Table 1.1).

Fatty acids are often grouped into two main categories based on the number of hydrogens attached to each carbon in the aliphatic chain. In saturated fatty acids each carbon is bound to two hydrogens and the two adjacent carbons in the chain. They are categorized as 'saturated' because no double bonds exist in the carbon chain. If at least one double bond between carbons is present the fatty acid is considered unsaturated because the carbon chain does not contain the maximum hydrogen capacity. In many cases individual fatty acids in the same saturation category have vastly different effects on health.

Saturated fatty acids

Saturated fatty acids are produced through de novo lipogenesis from glucose and other sources of acetyl-CoA. Fatty acid synthesis occurs in the cytoplasm when high levels of citrate and ATP are present. Fatty acid synthase (FAS) is the primary enzyme involved in fatty acid synthesis. It contains seven catalytic domains which correlate to seven steps of de novo fatty acid synthesis. Palmitate (16:0) is the product of de novo lipogenesis and is the most prominent saturated fatty acid in plants and animals. Plants can also produce shorter chain saturated fatty acids, such as lauric (12:0) and myristic (14:0) acids. Palmitate, whether synthesized in vivo through de novo lipogenesis or consumed in the diet, readily undergoes elongation to yield the saturated fatty acid stearate (18:0). High intake of dietary saturated fatty acids has been associated with obesity,

cardiovascular disease and diabetes in humans. Some of the basis for this association has been attributed to the ability of saturated fatty acids to promote inflammation and insulin resistance, as well as to increase adipose accumulation and risk of obesity. Palmitic acid has also been shown in cell models to upregulate the pro-inflammatory gene cyclooxygenase-2 as well as other pro-inflammatory cytokines through the activation of the transcription factor nuclear factor-kB. Other inflammatory markers such as C-reactive protein and interleukin-6 have also been reported to increase with palmitic acid intake (Fernández-Real, Broch, Vendrell, & Ricart, 2003).

Mono-unsaturated fatty acids

As the name suggests mono-unsaturated fatty acids (MUFA) contain just one double bond in the aliphatic chain. Mono-unsaturated fatty acids comprise an estimated 36% of total fat intake in Americans and 12% of total energy intake (NHANES, 2009-2010). For many humans, the most consumed fatty acid is oleic acid (18:1 n-9), a MUFA found in many foods such as olive oil (70%) and canola oil (60%). (Kris-Etherton, Eckel, Howard, Jeor, & Bazzarre, 2001). While oleic is often consumed, it is also synthesized by the desaturation of stearate (18:0) at the Δ9 carbon (Calder, 2015). Oleic acid has been shown to have anti-inflammatory properties (Yaqoob, 2002) slightly lower blood pressure, and improve glucose sensitivity (Bermudez et al., 2011). Effects are modest and mostly observed in intervention studies where oleic acid replaces saturated fats (Pérez-Martínez, Garcia-Rios, Delgado-Lista, Pérez-Jiménez, & López-Miranda, 2011). The effect of MUFA's on fat deposition varies by tissue and degree of obesity. In intervention studies more obese individuals saw greater weight loss when consuming a MUFA enriched diet than less obese individuals. Likewise, subcutaneous fat was reduced, while no effect was seen in perivisceral fat (Garaulet et al., 2001).

Poly-unsaturated fatty acids

Fatty acids with more than one double bond in the carbon chain are referred to as poly unsaturated (PUFA). Polyunsaturated fatty acids are consumed in the diet and are also synthesize in vivo by desaturase enzymes that catalyze the addition of a double bond to existing fatty acid chains. The position of the first double bond lends unique properties to PUFA that impact their physiological effects in the body. Any PUFA where the first double bond from the carboxylic acid group in the carbon chain is on the third carbon is considered an n-3 PUFA, while those with the first double bond on the sixth carbon are classified as n-6. The most commonly consumed n-6 PUFA in humans and animals is linoleic acid (18:2 n-6), which is an essential fatty acid produced by plants and found in abundance in nuts, seeds, and plant oils. Maize and soybean oil are made of 50-55% linoleic acid. Food sources, primarily animal products, also contain the longer chain n-6 PUFA species arachidonic acid (20:4 n-6). Dietary sources of n-3 PUFA are less common than n-6. Alpha linolenic acid

(LNA, 18:3 n-3) is also an essential fatty acid and is the most common dietary n-3 PUFA species. Common oils high in LNA are chia seeds (64%) and flaxseed oil (50%). The longer chain n-3 PUFA species eicosapentaenoic acid (EPA; 20:5 n-3) and docosahexaenoic acid (DHA; 22:6 n-3) are also found in animal and human diets, but to a lesser extent than LNA. These two fatty acids are commonly found in fish oil and other oils of marine origin. Alpha-linolenic can be converted to EPA and to a lesser degree DHA by elongation and desaturation proteins. The conversion of LNA to EPA is inefficient, but is more efficient than conversion to DHA. EPA and DHA are the predominant fatty acids in fish oil. Interestingly enough, fatty fishes rich in LC-PUFA are not efficient at synthesizing them. Most of the EPA and DHA is dietary from consumption of algae in lower trophic levels. Algal species synthesize EPA and DHA more efficiently because their genomes contain a more diverse set of the elongase and desaturase enzymes than vertebrates. Depending on the species, algae use different mechanisms to synthesize EPA, DHA and other very long chain PUFA from shorter and more saturated fatty acid substrates. (Khozin-Goldberg, Iskandarov, & Cohen, 2011).

The classes of n-6 and n-3 PUFA are relevant to health in humans and animals. The increased rates of obesity in the past the 30 years have been paralleled by increased n-6 and decreased n-3 in Western diets (Simopoulos, 2016). The typical American today consumes up to 1:30 (n-3:n-6) ratio, but when n-3 is increased, lowering the n-6: n-3 ratio, it seems to negate the deleterious effect of high n-6 consumption (Blasbalg, Hibbeln, Ramsden, Majchrzak, & Rawlings, 2011). At the cellular level, differential effects of n-6 vs. n-3 PUFA can result from differences in the sets of eicosanoids, prostaglandins, prostatyclins and other bioactive lipid metabolites that are produced from these two classes of fatty acids. For example, AA (20:4 n-6) is metabolized by cyclooxygenase, lipoxygenase, and cytochrome P450 pathways to produce eicosanoids and prostacyclins that, in general, are proinflammatory. In contrast, EPA can be metabolized to produce resolvins, which are considered to be anti-inflammatory (Martha H. Stipanuk & Caudill, 2013). Interestingly, the same enzyme (cyclooxygenase (COX)) is responsible for the initial step in AA and EPA metabolism, and has preferential affinity for AA. Therefore the relative ratio of these two fatty acids in the diet or in tissues is important.

n-3 PUFA, EPA, DHA, and adipose tissue.

Intuitively, it might be expected that pathways controlling the differentiation and metabolism of adipocytes, the body's depot for lipid storage, are influenced by different types of fatty acids and their metabolites. Epidemiological data in humans and experimental evidence in animals, including chickens, and from cells in culture confirm this relationship. In humans, a high ratio of n-6:n-3 PUFA content in the diet is associated with higher BMI than n-3 enriched diets (Garaulet et al., 2001). Similar relationships have been shown experimentally in chickens. For example chickens consuming diets enriched with flaxseed oil, high

in n-3, for 21 days had significantly less abdominal adipose than birds consuming diets enriched with lard, high in n-6 (Torchon, Das, & Voy, 2015). Several mechanisms may contribute to differential effects of n-6 and n-3 PUFA on adipose deposition. Eicosanoids and prostaglandins have been shown to affect the proliferation of preadipocytes and their differentiation into mature adipocytes (Negrel, Grimaldi, & Ailhaud, 1981). In addition, specific LC n-3 PUFA can act as ligands to activate PPARG as well as PPAR-alpha (PPARA), resulting in transcriptional effects on genes that control adipogenesis and fatty acid metabolism in adipocytes. With regard to adipose tissue, diets enriched in n-3 PUFA have been shown experimentally to reduce fat accretion in chickens and other species compared to diets with higher content of n-6 PUFA. Additionally, proliferation of mature adipocytes may be attenuated by LC-PUFA's incorporation into the cell membrane. Increasing LC-PUFA concentrations alters the membrane bound ARA phospholipid ratio leads to changes in eicosanoid biosynthesis. EPA outcompetes ARA and binds to cyclooxygenase (COX), leading to a reduction in PGE¬2, a major contributor to cellular proliferation. Reducing PGE¬2 increases incorporation of LC-PUFA into the cell membrane of pre adipocytes. A similar effect on differentiation occurs when the inducers of adipogenesis 15-doxy-J2 and PGD2 are reduced by increased competition for COX. A reduction of PGI2, an adipocyte released molecule which has pro adipogenic effects on preadipocytes, is also observed (Flachs, Rossmeisl, Bryhn, & Kopecky, 2009)

Cellular metabolism is also modified by the LC n-3 PUFAs EPA and DHA which increases mitochondrial biogenesis in the liver, more so than adipose tissue, through stimulation of AMPK. Phosphorylation of acetyl-CoA by AMPK reduces malonyl-CoA. Malonyl-CoA is a known inhibitor of carnitine palmitoyltranserase-1, a crucial mitochondrial transporter for β oxidation. Stimulation of AMPK by EPA and DHA leads to increased β oxidation as well as inhibition of lipogenesis. In adipocytes AMPK can be stimulated by both the adipokines leptin and adiponectin (Flachs et al., 2009). Normalization of leptin and adiponectin levels of rats fed a sucrose rich diet for nine months was reported after intervention EPA and DHA for two months. Reversal in insulin resistance and reduced adipose tissue hyperplasia (Rossi et al., 2005).

Relatively recent studies in animal models and humans suggest that either the ratio of n-3:n-6 PUFA or the abundance of n-3 PUFA species may be a means to reduce early deposition of excess adipose tissue in children and in animals, including broiler chickens. Rudolph et al. (2017) used a novel transgenic mouse model to show that increasing the n-3:n-6 ratio in the maternal blood during pregnancy significantly reduces adipose development in mice after birth. In humans, maternal fatty acid profiles in blood serum have been shown to impact birth weight and childhood fat deposition. One study which tracked maternal n-6 fatty acid serum concentrations and child fatness over time reported offspring fat mass was predicted by maternal n-6 poly unsaturated fatty acids (PUFA) concentration during pregnancy (Moon et al., 2013). The n-6 PUFA

dihomo-γ-linolenic acid (DGLA) in maternal serum is associated with higher BMI and waist circumference in 7 year olds (de Vries et al., 2014). Similarly, another study which examined n-6 and n-3 maternal serum levels reported that low n-3 and high n-6 concentrations are associated with higher body fat. Interestingly, in the same study as n-3 concentrations increased total body fat declined, but there was no change in BMI (Vidakovic et al., 2016). In addition, low maternal circulating DHA levels were associated with low birth weight in babies, suggesting DHA can act as a regulator of infant weight (Meher, Randhir, Mehendale, Wagh, & Joshi, 2016). The relationship between maternal levels of n-3 PUFA during pregnancy, specifically DHA and EPA, suggests that these fatty acids may reduce fat mass in offspring through developmental programming of adipose development, much like many environmental chemicals and maternal obesity promote offspring obesity. Recently, Beckford et al. developed a novel model using broiler hens to directly test the hypothesis that enriching the maternal diet in EPA and DHA attenuates adipose accretion after birth (hatch). Broiler-breeder hens were fed diets in which fat (2.3%, wt:wt) was supplied from either corn oil or fish oil. After hatch, all chicks were fed a standard commercially-formulated starter diet. At both 7 and 14 d of age, chicks hatched from hens fed the fish oil diet had significantly less adipose tissue, but normal body weights, compared to corn oil chicks. Reduced fat mass was paralleled by smaller but more adipocytes, and by changes in expression of genes and proteins that control adipogenesis and lipid metabolism. This study demonstrates that enriching developing adipose tissue in EPA and DHA can reduce fat development through multiple components of adipose growth.

Why EPA/DHA and not their precursor (18:3 n-3)?

A significant body of literature, including studies in the Voy lab, demonstrate that dietary EPA and DHA are potentially anti-obesogenic fatty acids. These two fatty acids are available in nature via marine-derived oils, but two features make them less than desirable for widespread use, particularly in the poultry industry which produces millions of broilers each year: 1.) they are relatively expensive compared to other plant-derive oils, and 2.) they are prone to oxidation in storage and in diets prior to consumption. A potentially viable alternative solution would be to increase the endogenous synthesis of EPA and DHA, which would alleviate both negative issues with fish oil.

Health benefits associated with ALA consumption predominantly come from its conversion to EPA and DHA. A number of enzymes are responsible for conversion including ELOVL2, ELOVL5, and Δ6 desaturation. Regulatory enzymes which control the n-3 elongation and desaturation responsible for EPA and DHA synthesis are in direct competition with n-6 fatty acids. With the low n-6: n-3 ratio consumed by western diets the less advantageous n-6 fatty acids outcompete n-3 fatty acids for elongation enzymes leading to a low conversion rates to EPA and DHA. Higher conversion rates from ALA to EPA and DHA have been reported in mouse and human embryo and infant models but these rates

drop rapidly after infancy (Jensen, Chen, Kennard Fraley, Anderson, & Heird, 1996). It has been theorized the higher conversion rates in early development are a biologic necessity for proper neuronal and ocular development (Makrides, Neumann, Byard, Simmer, & Gibson, 1994).

Fatty acid synthesis, elongation and desaturation

Both fatty acids derived from the diet and palmitate that is synthesized endogenously can undergo elongation and desaturation. Each elongase enzyme has specific substrates and adds two carbons at each elongation step. Fatty acids are elongated in the endoplasmic reticulum by a family of enzymes named very long chain fatty acid (ELOVL) proteins. Seven ELOVL genes (ELOVL2-7) have been identified in the chicken genome. Each ELOVL enzyme catalyzes the same functional reaction – addition of a two carbon subunit to a fatty acid chain. The enzymatic mechanism is similar to that of fatty acid synthase, which sequentially condenses 2-C subunits to synthesize palmitate. However each ELOVL enzyme is selective for a specific fatty acid substrate(s) and produces a select product(s). The substrates and products have chain lengths up to 34 carbons, although fatty acids longer than 24 carbons are rare in most mammals.

The metabolic conversion of α-linolenic acid to EPA and DHA is feasible but conversion rates are low. The pathway shares enzymes with linoleic acid metabolism, creating a competitive metabolic environment. People consuming western diets intake more linoleic than α-linolenic, a typical ratio being 7:20, which is a possible explanation as to why α-linolenic acid conversion rates to EPA and DHA is limited (Calder, 2015). While rates are low, conversion of α-linolenic to EPA and DPA, an intermediate between EPA and DHA, are greater than conversion rates to DHA (Chan et al., 1993) in part due to the competition for Δ6 desaturation by fatty acid desaturase 2 (FADS2) and also competition for elongation by ELOVL genes which are responsible for elongating fatty acid chains past 16 carbons (Portolesi, Powell, & Gibson, 2007). The conversion rates are affected by age. Human infants have been shown to have a slightly higher conversion rate to DHA from α-linolenic, approximately 1%, than adults (Clark, Makrides, Neumann, & Gibson, 1992).

ELOVL family of elongases

If elongases are present at appropriate levels, exposure to shorter chain fatty acids could result in conversion to LC-PUFA such as EPA and DHA (Table 1.2). The cost of feeding LC-PUFA is high and not currently a viable option for producers. If elongases are able to efficiently convert shorter chain fatty acids to EPA and DHA embryonic exposure to shorter fatty acids could have similar effects as in-ovo exposure to EPA and DHA through maternal diet (Beckford et al., 2017).

Elongation of Very Long Chain Fatty Acid 2 (ELOVL2) was discovered because of its similarity to ELOVL3, the first ELOVL discovered. In mammals ELOVL2 is highly expressed in liver and testis (Tvrdik et al., 2000). It, along with

ELOVL5, is specific for PUFA substrates. In multiple species ELOVL2 has been shown to elongate C20 and C22 PUFA, including EPA. Therefore ELOVL2 catalyzes a final elongation step in the synthesis of DHA. Activity for C18 PUFA has been reported in some species, but not in chickens (Gregory, Geier, Gibson, & James, 2013). When ELOVL2 metabolizes EPA it predominantly forms 24:5(n-3) (Leonard et al., 2002). Diets enriched in flaxseed oil and algal DHA have shown downregulation of ELOVL2 in liver and adipose tissue (Neijat, Eck, & House, 2017). Genetic deletion of ELVOL2 in mice increased serum levels of 20:5n-3 and 22:5n-3, consistent with elongation of these substrates by this enzyme (Zadravec et al., 2011). Three years later the same group confirmed ELOVL2 deletion increased serum 22:4n-6 and 22:5n-3 as well as increased 22:6n-3 and 22:5n-6 expression of SREBP-1c (Pauter et al., 2014).

Elongation of Very Long Chain Fatty Acid 3 (ELOVL3) was the first gene in the Elovl family to be discovered. At the time it was originally named Cold-induced Glycoprotein of 30kDa (Cig30) due to its increased expression during cold exposure in mouse brown adipose tissue (Petr Tvrdik et al., 1997). It was later renamed ELOVL3 as a more apt description of its function. In mouse models it has been shown to elongate saturated fatty acids as well as monounsaturated fatty acids (MUFA) no longer than 24 carbons (Tvrdik et al., 2000). It is expressed in brown adipose tissue, liver and hair follicles in the skin (Petr Tvrdik et al., 1997).

Elongation of Very Long Chain Fatty Acid 4 (ELOVL4) elongates both saturated fatty acids and e very long chain PUFA's with aliphatic chains of 28 carbons or greater (Zhang, Kothapalli, & Brenna, 2016). One study which overexpressed ELOVL4 in cultured cells suggests the gene is necessary in the synthesis of saturated C28 and C30 as well as PUFA C28 through C38 (Brolinson, 2009).

Elongation of Very Long Chain Fatty Acid 5 (ELOVL5) in chickens has unique properties compared to those described in mammals. In human and rat models ELOVL5 has been shown to elongate C18-20 fatty acids, but lacks the ability to elongate C22 carbon chains as it does in chickens (Gregory et al., 2013). In chickens ELOVL5 can synthesize 24:5(n-3) from C18-22 fatty acids. Both ELOVL2 and ELOVL5 have the ability to elongate DPA (22:5,n6), a precursor to DHA. As an example broilers fed an enriched ALA diet had higher hepatic ELOVL5 expression than ELOVL2 and were reported to have elevated hepatic DHA, but not DPA or EPA (Gregory et al., 2013). Neither ELOVL5 nor ELOVL2 expression changed when dietary ALA increased (Gregory et al., 2013). The abundance of hepatic DHA indicates ELOVL5 could be acting on similar C20 substrates as ELOVL2 increasing the ability to synthesize EPA and DHA in chickens. Relative abundance of PUFA specific elongases ELOVL5,2 in the liver of chickens may also synthesize DHA more efficiently than other terrestrial species.

Elongation of Very Long Chain Fatty Acid 6 (ELOVL6) ELOVL6 is reported to elongate palmitic (16:0) and palmitoleic (16:1) acid to stearic (18:0) and oleic

(18:1) acid by adding an additional two carbons to the aliphatic chain. Mice have been shown to have high expression of ELOVL6 in the liver and adipose tissue. In addition, ELOVL6 has been linked with obesity-induced insulin resistance in mice (Matsuzaka et al., 2007).

Elongation of Very Long Chain Fatty Acid 7 (ELOVL7) is the most recently discovered elongase. Most of the work done on ELOVL7 is in relation to cancer, specifically prostate cancer. This novel elongase has been shown to have highest specificity for elongation of ALA (18:3 n-3), which indicates that it could play a potential role in synthesis of EPA and DHA. In addition to attenuated prostate cancer growth, disruption of ELOVL7 activity was shown to significantly reduce levels of 20-C very long chain unsaturated fatty acids, suggesting it is involved in their synthesis (Tamura et al., 2009). An isoform of ELOVL7 has been identified in the chicken genome but not characterized.

Fatty acid desaturation

Stearoyl-CoA Desaturase (SCD) adds double bonds to saturated fatty acids between 12 and 19 carbons. SCD is the most well characterized of its isoforms. While it is expressed ubiquitously, elevated liver expression can be induced by feeding a high carbohydrate or saturated fat diet. The gene is also post-translationally regulated by proteases and the proteasomal pathway (Sampath & Ntambi, 2011).

Stearoyl-CoA Desaturase 5 (SCD5 is distinct from the 4 SCD isoforms as it has a higher GC content. It was once thought to only be expressed in humans and primates and went by the name hSCD5. It is highly expressed in the brain and though to play an important role embryonic development (Wang et al., 2005). In 2011 Gamarra and others discovered its expression in bovine adipose tissue as well as its inverse correlation with SERBP1 expression (Gamarra, Arakawa, Aldai, & López-Oceja). While expression has been characterized in chicken adipose, highest expression is in the brain and pancreas (Lengi & Corl, 2008) but its expression and role in embryonic development has not been characterized.

Fatty Acids Desaturase (FADS2) has the ability to induce double bonds at Δ4,6, and 8 by acting on a variety of substrates. Desaturation at Δ4 occurs only in 22 carbon fatty acids; Δ6 occurs in 16, 18, and 20 carbon chained fatty acids; and Δ8 desaturation is only observed in 20 carbon chains. Unlike most enzymes which desaturate PUFAs, FADS2 also has the ability to add a double bond to 16:0 (Zhang et al., 2016). The Δ6 desaturation of linoleic and α linolenic acid by FADS2 is believed to be one of the rate limiting reactions in the biosynthesis of EPA and DHA (Zhang et al., 2016).

Fatty Acid Desaturase 6 (FADS6) is less well-known than the other desaturase enzymes. It has been identified but not fully functionally characterized in human, mouse, and chickens.

Appendix

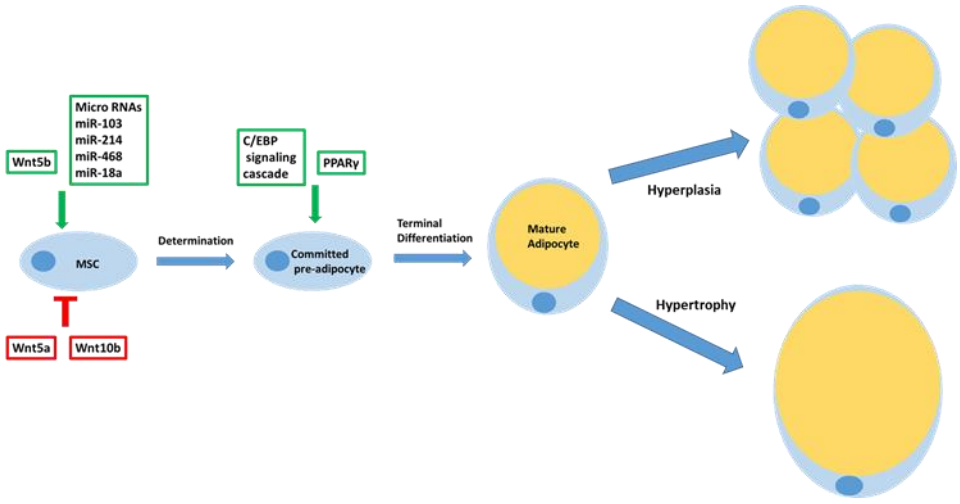

Figure 1.1 Known regulators of adipogenesis from mesenchymal stem cells. Adapted from (Uccelli, Moretta, & Pistoia, 2008).

Table 1.1 Consumption of Fatty Acids in the United States.

Common name	Systematic name	Abbreviation	Consumption (g)
capric	decanoic	10:00	0.59
lauric	dodecanoic	12:00	0.95
myristic	tetradecanoic	14:00	2.59
palmitic	hexadecanoic	16:00	16.77
stearic	octadecanoic	18:00	7.5
palmitoleic	9-hexadecenoic	16:01	1.28
oleic	9-octadecenoic	18:01	29.85
gadoleic	11-eicosaenoic	20:01	0.39
linoleic	9,12-octadecadienoic	18:02	19.32
a -linolenic	9,12,15-octadecatrienoic	18:03	2
arachidonic	5,8,11,14-eicosatetraenoic	20:04	0.19
EPA	5,8,11,14,17-eicosapentaenoic	20:05	0.03
DPA	7,10,13,16,19-docosapentaenoic	22:05	0.03
DHA	4,7,10,13,16,19-docosahexaenoic	22:06	0.07

Adapted from (NHANES, 2013-2014).

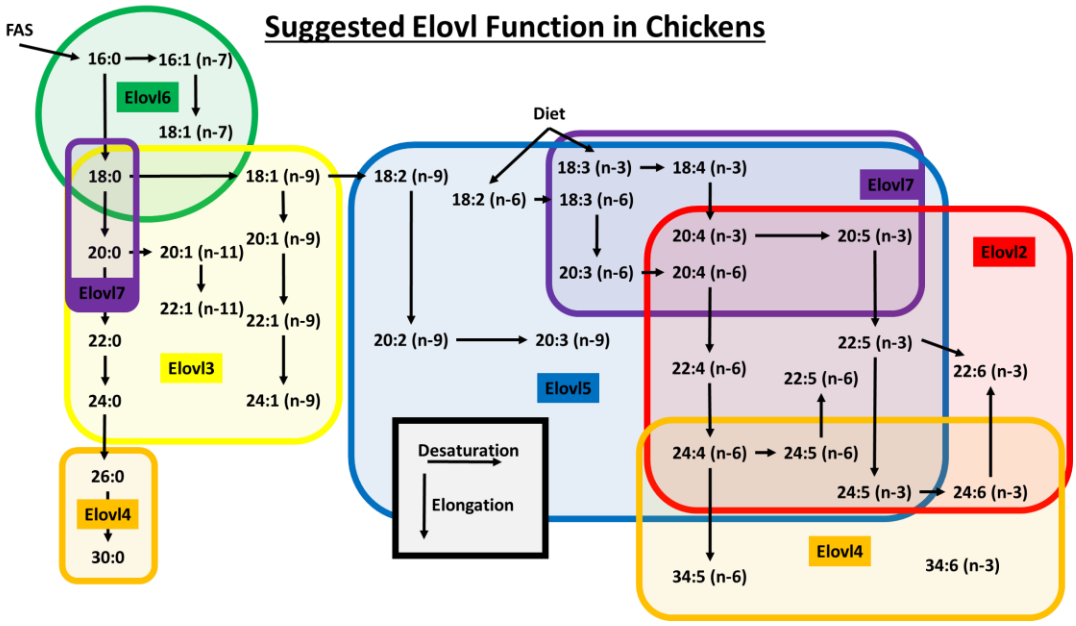

Figure 1.2 Suggested substrates and products of Elovl2-7 in broiler chickens. Adapted from (Brolinson, 2009).

Table 1.2 Elongase and desaturase characteristics.

Gene	Chr[1]	Exons[1]	AA[1]	Substrates[2]	Primary sites of expression[3]
ELOVL2	2	8	297	20:4 (n-3;n-6) 20:5 (n-3) 22:4 (n-6) 22:5 (n-3;n-6)	Testis, liver, brain, kidney, white adipose tissue (WAT)
ELOVL3	6	4	252	18:0 18:1 (n-9) 20:0 20:1 (n-9;n-11) 22:0 22:1 (n-9)	Liver, skin, brown adipose tissue (BAT), WAT
ELOVL4	3	7	314	24:4 (n-6) – 32:4 (n-6) 26:0 28:0	Ubiquitous
ELOVL5	3	8	295	18:2 (n-9) 18:3 (n-6) 18:4 (n-3) 20:4 (n-3;n-6) 20:5 (n-3)	Ubiquitous
ELOVL6	4	4	265	16:0 16:1 (n-7)	Ubiquitous
ELOVL7	Z	8	279	18:0 18:3 (n-3;n-6) 20:4 (n-3;n-6) 20:5 (n-3)	Liver, kidney, pancreas, adrenal glands, and prostate
SCD	6	6	357	16:0 18:0	Liver, BAT, WAT
SCD5	4	6	330	16:0 18:0	Brain, pancreas, WAT
FADS2	5	13	444	18:2 (n-6) 18:3 (n-3)	Liver, WAT
FADS6	18	6	405	Unknown	Liver

[1] Based on the chicken genome (Gallus_gallus 5.0, ensemble.org)
[2] Based on studies in chicken, rodents and humans
[3] Adapted from (Brolinson, 2009).

CHAPTER 2
DEVELOPMENTAL REGULATION OF ELONGASES AND DESATURASES IN BROILER CHICKS AND EMBRYOS

The authors of the work presented in this chapter are planning on submitting it for publication post-graduation.

Abstract

Broiler chickens are an attractive model to study early onset childhood obesity in humans due to their ability to synthesize and mobilize fatty acids before hatching. Chickens contain multiple enzymes involved in the elongation and desaturation of shorter chain fatty acids to long chain poly-unsaturated fatty acid particularly eicosapentaenoic acid and docosahexaenoic acid. Consumption of long chain poly-unsaturated fatty acid, commonly known as fish oil, have been shown to be related to multiple health effects including decreased inflammation and decreased rates of heart disease. Maternal consumption of eicosapentaenoic acid and docosahexaenoic acid in pregnant mammals has been shown to reduce childhood adipose development. In chickens embryonic exposure has reduced hypertrophy and increased hyperplasia of adipocytes. This study observes the broiler chickens ability to synthesize eicosapentaenoic acid and docosahexaenoic acid throughout embryonic and post hatch development in adipose tissue. Targeted RNA sequencing was used to characterize the expression of the elongase and the desaturase families of genes in abdominal, subcutaneous, and crop adipose tissue as well as in the liver of embryonic and perinatal chicks -8, -6, -4, +7, +14 days from hatch. The study confirms the ability to synthesize eicosapentaenoic acid and docosahexaenoic acid does change throughout development.

Introduction

Genetic selection for rapid growth inadvertently increased adipose tissue deposition in commercial broiler chickens. As an example, chickens from the Cobb 500 broiler line have approximately three times the amount of adipose tissue at harvest when compared to unselected broiler chickens (Collins, Kiepper, Ritz, McLendon, & Wilson, 2014). Excess adiposity in broiler chicks is a multifaceted problem affecting producers, consumers, and animal well-being. Excess adipogenesis requires nutrients from feed which producers would prefer go toward muscle production. With feed constituting 70% of the input cost for poultry rearing, reducing adipose deposition would be economically beneficial to the poultry industry (Foods, 2017). In addition to effective wasting of feed, excess fat also compromises fertility and well-being in broiler breeder hens. Finally, the excess fat around market parts must be trimmed off at harvest in order to be packaged and sold to consumers, which increases time and labor costs for the processing facility. Therefore, for multiple reasons, reducing fatness of broilers would benefit the poultry industry.

Long chain poly unsaturated fatty acids (PUFAs), specifically the n-3 species eicosapentaenoic acid (EPA; 20:5 n-3) and docosahexaenoic acid (DHA; 22:6 n-3), have been shown to reduce excess adiposity in a number of species (Kelly, Gilman, Kim, & Ilich, 2013). In vitro studies have shown EPA and DHA

inhibit adipocyte differentiation suggesting they may be effective in reducing the initial deposition of adipose tissue during development (Howard et. al., 2016.) EPA has been shown to reduce lipid droplet size as well as total lipid accumulation in-vitro (Manickam, Sinclair, & Cameron-Smith, 2010). Another study showed a reduction in adipocyte size in broilers fed n-3 fatty acids when compared to those fed tallow based diets (González- Ortiz, Sala, Cánovas, Abed, & Barroeta, 2013).

The use of EPA and DHA is a promising prospective for fat reduction in broiler chicks, but there are substantial challenges to implementation. When EPA and DHA levels exceed 5% chicken meat takes on a fishy flavor and spoils more quickly. The taste is unaffected at concentrations below 5% (Miller & Robisch, 1969). Also, fish oil, typically used to provide EPA and DHA, greatly exceeds the cost of conventional soybean oil used in most feeds (Nyquist, Rødbotten, Thomassen, & Haug, 2013). The fatty acids EPA and DHA are synthesized by elongation and desaturation of the precursor linolenic acid (ALA; 18:3 n-3). Theoretically, therefore, an alternative strategy to provide EPA and DHA would be to enrich the diet in ALA, which is abundant in flaxseed and other seed oils. In mammals, the elongation to ALA to EPA is inefficient due to low levels of elongase activity. Avian elongases have distinct features and may be more efficient in conversion of ALA to EPA. Some studies have demonstrated enriching the broiler diet in ALA reduces adipose mass at market age, although inconsistent results have been reported (Ferrini, Baucells, Esteve-Garcia, & Barroeta, 2008). Supplementation of foods rich in ALA has been shown to increase thermogenesis and have slight fat reducing properties (Shin & Ajuwon, 2018).

In general, studies testing the effects of various diet manipulations on broiler adiposity initiate experimental diets at two to three weeks of age. From this point until market age (~ 42 days), adipocyte hypertrophy is the primary means of adipose accumulation. Previous studies have shown dietary fish oil and, to a lesser extent flaxseed oil, reduce adiposity and adipocyte size and inhibit adipocyte differentiation when fed to chicks just after hatch (Howard, 2016). Beckford and others targeted an even earlier stage by enriching the diet of the hen in EPA and DHA (Beckford et al., 2017). Chicks hatched to hens fed a fish oil-enriched diet had significantly reduced adipose tissue and smaller adipocytes than chicks hatched from hens fed a corn oil-enriched diet. If the elongase and desaturase enzymes necessary to convert ALA to EPA are sufficiently abundant and active during development, it may be possible to produce similar benefits from embryonic exposure to ALA. However, little is known about the developmental expression patterns of the elongase and desaturase enzymes in the developing chick.

The synthesis of EPA and DHA through the Elongation of Very Long Chain Fatty Acid enzymes ELOVL2 and ELOVL5 is well characterized in mammals and avians. In this process α-Linolenic acid (18:3n-3) undergoes alternating desaturation and elongation to synthesize EPA and DHA (Jing,

Gakhar, Gibson, & House, 2013). An alternate synthesis pathway utilizing ELOVL7 has also been described (Tamura et al., 2009). In this process α-Linolenic acid would first undergo an elongation by ELOVL7 then be desaturated to synthesize EPA. Another elongation by ELOVL7 would synthesize Docosapentaenoic acid (22:5) followed by another desaturation to form DHA (Tamura et al., 2009). Synthesis of EPA and DHA by the ELOVL7 pathway is less characterized than by the ELOVL2 and ELOVL5 pathway.

Chen and others observed the preferential mobilization of long chain fatty acids prior to hatching (Chen, Suh, Choi, Shin, & Lee, 2014). It is thought the mobilization of fat before hatching is to supply energy for the demanding hatching process. At hatch chicks go through a rapid dietary change in fatty acid supply. More than 90% of an embryonic chicks' energy comes from fatty acids in the yolk (Nobel & Cocchi, 1990). After hatching, a chicks diet contains <5% fat. Jing and others in 2013 found dietary changes in fatty acids manipulate expression of ELOVL2, and ELOVL5, enzymes responsible for endogenous EPA and DHA synthesis, in the liver. They also observed ontogenic regulation of ELOVL2 and ELOVL5 in the liver after hatching (Jing et al., 2013). To our knowledge the expression of genes associated with fatty acid elongation and desaturation has not been observed during the dynamic dietary change at hatch.

The hypothesis of this study is that elongases and desaturases are developmentally regulated in pre- and post-hatch chick adipose tissue. The objective are firstly to define developmental patterns of expression of known avian elongases and desaturases within and between adipose depots in broiler chicks.

Materials and methods

Animals and housing

All animal procedures were conducted under guidelines of the University of Tennessee Animal Care and Use Committee. Fertilized broiler eggs were acquired from a commercial hatchery in Chattanooga, TN and incubated using standard incubation methods. Hens were fed a standard commercial parent stock diet for broilers containing 1.25% linoleic acid. Eggs were incubated (99.5^{o}F and 60% humidity) for 19 days then transferred to a hatcher (98^{o}F and 66% humidity) and allowed to hatch. Chicks were brooded, housed in standard conditions (90-95^{o}F), and fed a commercial chick starter diet (3% crude fat).

Sample collection

Subcutaneous adipose tissue (SQ) was collected from chicks at ages -8, -6, -4, +7, and +14 days from hatch (E13, E15, E17, D7, and D14 respectively; n= 4-7), for RNA isolation and sequencing (described below). Liver was collected at E17, D7, and D14. Abdominal (AB) and crop adipose were collected at D7 and D14 because they develop after E17. Chicks were euthanized by asphyxiation with CO2. An incision was made with scissors from the vent along the bird's side

to expose the subcutaneous adipose tissue forming on the leg. Next, a cranial lateral incision was made from the vent to the esophagus to expose the abdominal and crop adipose tissue. Tissues were separated from viscera and connective tissue, placed in 15ml tubes, then snap frozen in liquid nitrogen. All tissue samples were stored at -80C.

RNA extraction

Total RNA was extracted from tissue samples (~ 50-100 mg) using TRIzol (Invitrogen™ Carlsbad, CA). Total RNA was quantitated spectrophotometrically by optical density at 260 nm using an Amersham Ultra Spec 1300 Pro Spectrophotometer (Amersham Buckinghamshire, England). Quality was verified visually by amplifying the 18s gene using traditional gel electrophoresis. The iScript cDNA synthesis kit (Bio-Rad Hercules, CA) was used to synthesize cDNA from ~ 500 ng of total RNA.

Targeted RNA sequencing

Primers were designed using NCBI Primer Blast (https://www.ncbi.nlm.nih.gov/tools/primer-blast) for the Elongation of Very Long Chain Fatty Acid (ELOVL2-7) gene family, Sterol-Coa Desaturase (SCD), SCD5, Fatty Acid Desaturase 2(FADS2), FADS6, Cell Death Activator (CIDEA), and Uncoupler protein 3 (UCP3; Table 2.1). Amplicons extended multiple adjacent exons to ensure desired product. Exons were selected based on the most identical exons compared between the chicken refseq genome (Gallus_gallus-5.0/galGal5) and mRNA from GenBank for each gene on the USCS Genome Browser. Reads were aligned to the reference genome using STAR. Individual readers were counted using HTSeq. Total counts were normalized by the geomean of housekeepers ACTB, TBP, and YWHAZ because these genes were the most stable across age and tissue type. Partial least squares discriminant analyses (PLS-DA) of adipose tissue were performed with MetaboAnalyst 4.0 (www.metaboanalyst.ca) using an X-axis component of 1, and a Y-axis component of 2.

Statistical analyses

A completely randomized design with nested treatment effects of age and tissue type within age was used to evaluate parameters. Statistical analyses were performed in SAS 9.4 (SAS Institute Inc., Cary, NC, USA). Prior to statistical testing, assumptions of ANOVA including normality of residuals, equal variance, and independence of observations was checked using PROC UNIVARIATE diagnostics. As needed, natural log transformations were used for non-normal samples and reads. In the case of 0 expression observations, a value of 0.1 was added to observations prior to transformation. Mixed Model ANOVA within PROC GLIMMIX were used to compare differences in gene expression between age categories and the nested effects of tissue type within

age for qPCR and RNAseq data. Statistical significance was set at $p \leq 0.05$ followed by post-hoc mean separation to identify differences between groups.

Results

Adipose tissue development begins in the embryo around E12 when fatty acids from the yolk begin to be mobilized and stored in subcutaneous adipocytes located in the parafemoral area. All observed elongases and desaturases were expressed in SQ adipose tissue in the parafemoral area at all ages. Of the elongases, ELOVL7 had the highest mean expression. The least expressed genes were ELOVL2, 3, 4. ELOVL5, 6 showed moderate expression. As shown in Figure 2.1 ELOVL2, 3, 5, are minimally expressed at embryonic ages, maximally expressed at D7, then decrease expression at D14 ($p < 0.0001$). ELOVL4 shows minimal expression at E13 and E15, peak expression at E17, then decreases at D7 and D14 ($p < 0.0001$). ELOVL6 decreases with age ($p < 0.0497$). ELOVL7 increases slightly as embryos age, dramatically decreases expression at D7, then is maximally expressed at D14 ($p < 0.0001$).

Desaturases were expressed in SQ adipose at all ages. As seen in Figure 2.2 FADS6 showed lowest expression of the desaturases. FADS2 showed a similar expression pattern as ELOVL2, 3, 5. FADS2 was minimally expressed in embryonic tissues, peaked at D7, then decreased at D14 ($p < 0.0001$). FADS6 expression decreased from E13 to E15, increased at E17, then decreased at D7 and D14 ($p < 0.0001$). As with FADS2, SCD was expressed minimally at all embryonic ages, increased significantly at D7, then decreased at D14 ($p < 0.0001$). SCD5, in contrast, was maximally expressed in all embryonic ages, decreased significantly at D7, then increased at D14 ($p < 0.0001$). Dramatic shifts in expression were observed at D7 for all ELOVLs excluding ELVOL4, 6 and desaturases, except FADS6.

The abdominal adipose depot (AB) becomes visible after hatching in broiler chicks. All elongases and desaturases observed in this study were expressed in AB adipose tissue at all ages. Of the elongases, ELOVL7 had the highest expression. The least expressed genes were ELOVL2, 3, 4. ELOVL5 and 6 showed moderate expression. As shown in Figure 2.7 ELOVL2 and ELVOL5 expression decreased from D7 to D14 ($p < 0.0001$). ELOVL7 shows an opposite pattern of expression where it is lowest at D7 and increases at D14 ($p < 0.0001$). Desaturases were also expressed in AB adipose at all ages. FADS6 showed lowest expression of the desaturases, while all other desaturases were similarly moderately expressed. FADS6 decreased expression from D7 to D14 ($p < 0.0001$).

Overall liver had higher expression than AB adipose for ELOVL2, 5, 6, FADS2, FADS6, and SCD. Abdominal adipose was higher expressed than liver for ELOVL3, 4, 7, and SCD5. In liver tissue, ELOVL5 was the only gene upregulated from D7 to D14 ($p < 0.0001$). No other genes showed differential expression from D7 to D14. The elongases ELOVL2, 5, 6 were upregulated from E17 to D7 ($p < 0.0001$, < 0.0001, $p < 0.0497$ respectively). FADS2 and SCD were

upregulated in liver from E17 to D7 ($p < 0.0001$). SCD5 was downregulated at the same ages ($p < 0.0001$). There are more regulatory changes in the liver when comparing embryonic chicks to post hatched chicks than when comparing D7 to D14.

Overall, SQ adipose tissue and crop adipose tissue differed from each other at D7, but neither differed from AB adipose tissue. As seen in Fig 2.4 abdominal adipose tended to group with either crop or SQ, it did not have a unique expression pattern. By D14 the three adipose depots showed no difference in expression. The differences in adipose tissue is also seen by the differences in regulatory patterns of elongases and desaturases. Post hatch, D7 specifically, tended to show more differential regulation in SQ adipose than in crop. Based on expression levels SQ and AB adipose should have greater elongase and desaturase activity at D7 than crop adipose tissue.

Significant differences were seen with an age effect on ELOVL2, 3, 4, 5, 7, FADS, FADS6, PPARg, FASN, SCD, and SCD5 ($p < 0.0001$). No differential expression was observed with an age effect for ELOVL6. Letter grouping for significance is found in Table 2.4. ELOVL2 had lowest expression at E13 and E15, increased at E17 and D7, then decreased at D14. Expression of ELOVL3 did not differ at any embryonic ages, increased at D7, then decreased at D14. ELOVL4 expression was significantly less at E17 than E13, but E15 did not differ either embryonic ages. Perinatal expression of ELOVL4 was less than embryonic ages. ELOVL5 expression did not differ from E13 to E15, decreased at E17, increased to its highest expression at D7 then decreased again at D14. Expression of FADS showed lowest expression at all embryonic stages, was expressed highest at D7, and then decreased at D14. Expression of FADS6 did not differ between E13, E15, and D7. FADS6 showed highest expression at E17 and Lowest at D14. E13 expression of FASN did not differ between E15 and D14. Lowest expression levels were observed at E17, and highest expression at D7. Expression of SCD did not differ between E13 and E15, decreased at E17, and then increased at D7. SCD D7 values and D14 values did not differ. The expression of SCD5 showed highest expression at E13 and E15, decreased at E17, and then decreased again at D7. Expression increased from D7 to D14 for SCD5. PPARg showed no difference in expression between E13 and E15, decreased at E17, then increased D7 and D14.

Discussion

Synthesis of EPA and DHA from their precursor linolenic acid (18:3 n-3; LNA) requires both elongation and desaturation. The classical pathway for EPA synthesis from LNA begins with a desaturation to 18:4 n-3, catalyzed by Δ6 desaturase, followed by elongation to 20:4 n-3 then a second desaturation to produce EPA. The elongation steps of this pathway have been attributed to ELOVL2 and ELOVL5. An alternative pathway has also been described in which ELOVL7 catalyzes the synthesis of 20:3 n-3 directly from LNA. Further

desaturations synthesize EPA then ELOVL7 catalyzes the synthesis of DPA (22:5). Both pathways could therefore contribute to EPA and ultimately DHA synthesis in chick adipose tissue. Similarly to Atlantic Bluefin Tuna, low expression of elongases and desaturases will likely lead to less synthesis of EPA and DHA (Morais, Mourente, Ortega, Tocher, & Tocher, 2011). As in the case with mammals, infants and embryos have greater concentrations of EPA and DHA due to increased expression of elongases and desaturases. Though they have increased synthesis early in life, as individuals mature the ability to synthesize EPA and DHA decreases (Jing et al., 2013). In human and chicken models increased embryonic exposure to EPA and DHA has been shown to have anti-adipogenic properties which persist later in life (Beckford et al., 2017; Vidakovic et al., 2016).

Expression levels of elongases responsible for the synthesis of EPA and DHA in SQ tissue indicated the two pathways act at specific developmental stages. ELOVL7 was identified more recently than ELOVL2 and ELOVL5, and relatively little is known about its functional role beyond its substrate preference for LNA and EPA. Expression of ELOVL7 was relatively high in embryonic depots but then declined by ~ 30.2% in D7 post-hatch tissue, then increased ~25.7% at D14. The high expression of ELOVL7 suggests the capacity to synthesize EPA from LNA is highest at embryonic ages and D14. The lowest expression of ELOVL7 is at D7. Expression of ELOVL2 and ELOVL5 was low in embryonic subcutaneous fat, but increased sharply at D7 then decreased again by D14 ($p < 0.0001$). This suggest embryos use the ELOVL7 pathway to synthesize LC-PUFA then the classical pathway after hatch at D7. Synthesis then changes back to the ELOVL7 pathway at D14.

Expression changes could be driven by the change in dietary fatty acids at hatch. Embryonic chicks derive 90% of energy from fat in the yolk and post hatch chick diets contain approximately 3-5% fat (Nobel & Cocchi, 1990). The transition in nutrient utilization combined with the nutrient requirement for DHA in developing chicks could explain the increased expression of ELOVL2, ELOVL5, and FADS2. The decreased expression of PPARg in SQ adipose tissue along with the upregulation of ELOVL2, ELOVL5, and FADS2 suggests an increased EPA and DHA profile could come from previously stored, mobilized fatty acids. An upregulation of FASN at E17 and D7 indicates the elongases and desaturases could be acting on dietary sources. More work needs to be done to determine when the expression of elongase and desaturase genes is highest and if the elevated expression results in increased an increased EPA and DHA content. Further investigation to determine the origin of the EPA and DHA precursors is also needed.

When comparing SQ adipose tissue, all embryonic ages tended to group together with similar expression patterns. Significant differential expression was seen at D7 and D14 (Figure 2.3). Post hatch D7 chick expression differed more from embryonic ages than D14. At D7 SQ and Crop adipose tissue were dissimilar but neither differed from abdominal adipose. Individual AB fat samples

tend to be more like either SQ or crop fat, suggesting abdominal adipose tissue has the potential to either be more active in LC-PUFA synthesis at D7 similar to SQ fat, or less active like crop fat. Future studies will be needed to determine the mechanism driving the metabolic similarity of AB and SQ adipose. ELOVL5 and FADS2 were among the genes differentially regulated between the AB samples who grouped more with SQ rather than crop fat suggesting there is a mechanism enabling AB adipose to synthesize LC-PUFA similar to SQ adipose. At D14 all adipose samples behaved similarly in regards to gene expression. Neither AB nor crop fat differed between D7 and D14 (Figure 2.3).

Many factors could be responsible for driving the activity of SQ and AB fat deposits at D7, the most likely being the nutrient transition at hatch. The extreme metabolic transition from a 90% lipid diet to a mostly carbohydrate diet, 5% lipid, happens rapidly. Whereas the weaning process in mammals is often gradual, happening in days or weeks, precocial chicks make a similar metabolic transition when hatched in approximately two hours. The stress and rate of the extreme metabolic transition could be a driving factor in the genetic regulation seen at D7. Chicks have likely become adjusted to a carbohydrate based diet at D14 when downregulation occurs.

The targeted RNAseq dataset included a panel of genes that play various role in adipocyte development and metabolism. A correlation analysis was utilized to identify other genes showing similar patterns of expression as ELOVL7 and are potentially co-regulated at the expression level. A total of 63 genes were significantly correlated with ELOVL7 expression (fdr $p< 0.05$) in subcutaneous adipose tissue across ages. The set of 53 genes that showed a significant positive correlation included Cpt1a ($r2=0.6398$; fdr p-value = 1.02E-07) and Acox1 ($r2=0.62285$; fdr p-value = 2.77E-07), the two rate-limiting genes for fatty acid oxidation in mitochondria and peroxisomes, respectively (Table 2.1). The relationship between Acox1, Cpt1a, and ELOVL7 is interesting because genetic leanness in meat-type chickens is associated with higher levels of both adipose tissue EPA and DHA content and expression of genes regulating fatty acid oxidation. In addition, diets enriched in fish oil promote leanness and increase fatty acid oxidation in multiple species. The correlation between Acox1, Cpt1a, and ELOVL7 suggest ELOVL7 may play a role in upregulation fatty acid oxidation through its ability to generate EPA, and potentially DHA. EPA and DHA then activate PPARa which upregulates CPT1a and Acox1 expression leading to increased oxidation in mitochondria and peroxisomes (Figure 2.4).

As shown in Figure 2.1 and Figure 2.2, a subset of elongases and desaturases exhibited a spike in expression at 7d followed by a return to lower levels, comparable to those in the embryo, at day 14. At D7 chicks are adapting to a switch from a high fat to a high carbohydrate diet and are defending their body temperature against the cooler post-hatch environment. Of the genes which exhibited a spike in expression, reads for ELOVL3 were primarily detected at D7. In both embryonic and D14 adipose, ELOVL3 expression levels were in the 7th percentile, but the 18th percentile in 7d adipose. In mammals, expression of

ELOVL3 is associated with thermogenic brown adipose tissue (BAT), where it is thought to elongate fatty acids in BAT for preferential oxidation. Thermogenesis in avian adipose tissue is poorly described due to their lack of brown adipose tissue. Nonetheless, chicks are homeotherms and need to maintain body temperature. The upregulation of ELOVL3 post hatch suggests a beiging of white adipose tissue (WAT) to function thermogenically similar to BAT. Possible beiging is corroborated by the upregulation of other BAT genetic markers: CIDEA, UCP3, and various SLC25 genes 7 days post hatch (figure 2.6). SQ adipose tissue had the highest expression of ELOVL3, CIDEA, and UCP3 at D7 suggesting thermogenesis more likely to occur in SQ fat deposits rather than AB or crop deposits. Previous studies have shown CIDEA is significantly upregulated by acute fasting in adipose tissue from broiler chicks, suggesting an unappreciated thermogenic capacity of white fat (Howard, 2016).

Lowering the LA:ALA ratio in diets has been shown to increase expression of FADS1, FADS2, ELOVL2 and ELOVL5 as well as increase concentrations of EPA and DHA in broiler chickens over 21 days old (Jing et al., 2013). Future studies will need to be performed evaluating the impact of a lower LA:ALA exposure to developing broiler embryos and their impact on expression elongases and desaturases as well as LC-PUFA synthesis.

In summary, the capacity to elongate and desaturate fatty acids is developmentally regulated in broiler chicks. ELOVL7 may be the primary enzyme responsible for the elongation required to synthesize EPA and DHA from linolenic acid (18:3n-3). The pathway developing broilers synthesize EPA and DHA changes ontogenically. The ELOVL7 pathway is primarily used by embryos and D14 chicks. The classical ELOVL2 and ELOVL5 pathway is used after hatch at D7. The change in EPA and DHA synthesis pathway is likely driven by the extreme change in dietary fatty acids at hatch. At D7 SQ and crop adipose depots differ from eachother, but neither differ from AB adipose. At D14 all fat depots were similar. The upregulation of ELOVL3, CIDEA, and UCP3 at D7 indicates adipose tissue may be thermogenic after hatching. Despite the lack of brown adiposities, chicks may use thermogenesis in SQ adipose tissue to adapt to cooler temperatures after hatching.

ELOVL7 activity may be a target enzyme through which to increase endogenous EPA and DHA synthesis thus increasing the efficiency of broiler chicken by the reduction of adipose tissue. Similar studies may also yield healthier chicken by increasing EPA and DHA content for broiler chicken consumption.

Acknowledgments

I express my gratitude to Drs. Brynn Voy, Jennie Ivey, and Lannette Edwards for their valuable input and comments, Dr. Kurt Lamour for help with the RNA sequencing. I would also like to thank Suchita Das for technical help. I also thank The University of Tennessee Animal Science Department for education and funding.

Appendix

Table 2.1 Genes and primers used for targeted RNA sequencing.

Gene	Forward Primer	Reverse Primer	Amplicon Size (BP)
ELOVL2	GCCTCATGTTCCAGTCTTCC	TGCAGCTGTTCTTGAAGGTG	98
ELOVL3	CAAAGTCCTGGAACTGGGTGA	TGAGTGTGAGCATGTGGTGG	92
ELOVL4	CTGAACGACACCCTCGAGTT	TGGAAATGGAGACTGCATCA	90
ELOVL5	AGCGATGCGTCCTTATCTGT	CCACAGCTGGTCTGGAAGAT	90
ELOVL6	GGTGGTCGGCACCTAATGAA	GCACCGAATATACTGAAGACAGC	98
ELOVL7	AATTTGCTCCAGGTGGCTTA	CGGTCCCAAAGAACAGATTC	98
FADS2	CGGCAAGAAGAAGCTGAAGT	AAATACACGGGGATGAGCAG	90
FADS6	GCCTGTCTGACAACATGTGC	GCCGTAGGTGTCCTCATTGT	96
SCD	TCACATGTTTGGCAATCGGC	GTGGAAACCTTCTCCTAGGGC	91
SCD5	CACACCTTCCCCTTCGACTA	AACCCCAACCAAAACATGAA	100
UCP3	GTGGATGCCTACAGGACCAT	GCCGCAGTTAATGATGGAGT	99
CIDEA	CCTGCAAGAACTCATCAGCA	GTGTCCACAACTGTGCCATC	96
YWHAZ	GTTGCTGCTGGAGATGACAA	GTGTTGGCTGCATCTCCTTT	97
ACTB	GAACTCCCTGATGGTCAGGTC	CCACAGGACTCCATACCCAA	98
TBP	CCCTCAGGGTGCAATGACTC	GACTGTTGGTGCTCTGGACA	98
CPT1-A	GGAGACATCCACGAAGGAGA	AATACCAGCATTGGCAGTCC	99
ACOX	TCCGGCCGTTAAAGATACAG	TTTTGCAAGACCCTCCATTC	94
FASN	TGAAGACTTGCCTGGATGTG	TGGACACGAGAGAACAGACG	100

Table 2.2 Summary of ANOVA results comparing gene expression across ages and tissues.

Day	E17	7	14	7	14	7	14	E13	E15	E17	7	14	p value
Tissue	Liver	Liver	Liver	AB	AB	Crop	Crop	SQ	SQ	SQ	SQ	SQ	
SCD5	BC	D	D	C	C	AB	BC	A	A	D	C	C	<0.0001
SCD	E	A	ABC	ABC	AB	ABC	BC	D	D	A	C	C	0.0001
PPARG	A	A	A	A	A	A	A	A	A	A	A	A	1
FASN	G	A	AB	C	EF	F	EF	CD	DE	B	EF	EF	<0.0001
FADS6	A	A	A	CD	F	EF	EF	B	C	C	DE	DE	<0.0001
FADS2	E	A	A	C	CD	D	D	E	E	B	D	D	<0.0001
ELOVL7	EF	F	FG	DE	AB	ABC	A	CD	BCD	ABCD	G	ABC	<0.0001
ELOVL6	B	A	A	B	B	B	B	B	B	B	B	B	0.0497
ELOVL5	G	A	B	D	EF	EF	E	E	E	F	C	EF	<0.0001
ELOVL4	CDE	DE	E	C	C	CD	CD	AB	B	A	A	B	<0.0001
ELOVL3	EF	DEF	F	BC	CDEF	BCDE	DEF	BCD	BCDE	BC	A	B	<0.0001
ELOVL2	D	A	A	C	DE	DE	DE	F	F	EF	B	D	<0.0001

Letter grouping based on significance (p=0.05) where groups in a row with the same letter did not differ significantly.

Table 2.3 Summary of ANOVA results comparing gene expression in SQ adipose tissue across development.

	E13	E15	E17	7	14	p value
ELOVL2	D	D	C	A	B	<0.0001
ELOVL3	B	B	B	A	B	<0.0001
ELOVL4	A	AB	B	C	C	<0.0001
ELOVL5	C	C	D	A	B	<0.0001
ELOVL6	A	A	A	A	A	0.1416
ELOVL7	AB	AB	BC	A	C	<0.0001
FADS	C	C	C	A	B	<0.0001
FADS6	B	B	A	B	C	<0.0001
FASN	BC	B	D	A	C	<0.0001
PPARG	A	A	C	B	B	<0.0001
SCD	B	B	C	A	A	<0.0001
SCD5	A	A	B	D	C	<0.0001

Letter grouping based on significance (p=0.05) where groups in a row with the same letter did not differ significantly.

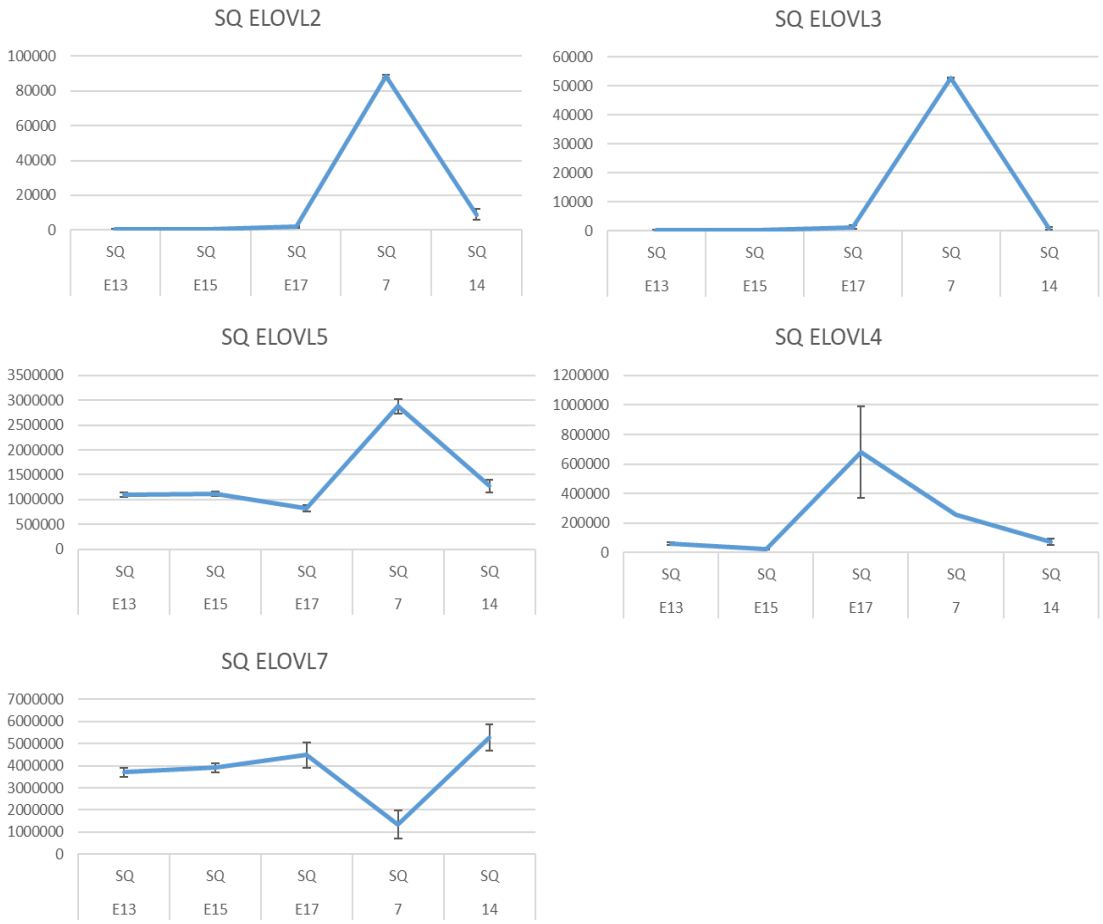

Figure 2.1 Developmental changes in expression of elongase genes (ELOVLs) 2, 3, 4, 5 and 7 in SQ adipose tissue.

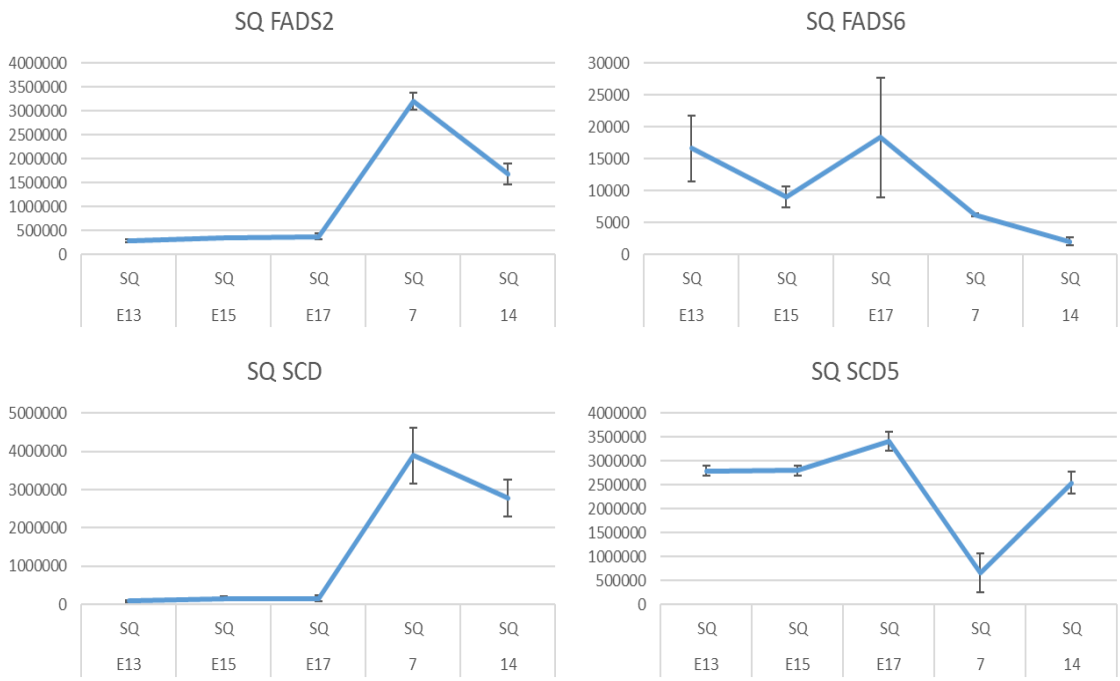

Figure 2.2 Developmental changes in expression of desaturase genes in SQ adipose tissue.

Values on the y-axes represent normalized total counts (mean +/- std dev) from targeted RNA sequencing; n=X-Y/age. FADS, fatty acid desaturase; SCD, stearoyl CoA desaturase.

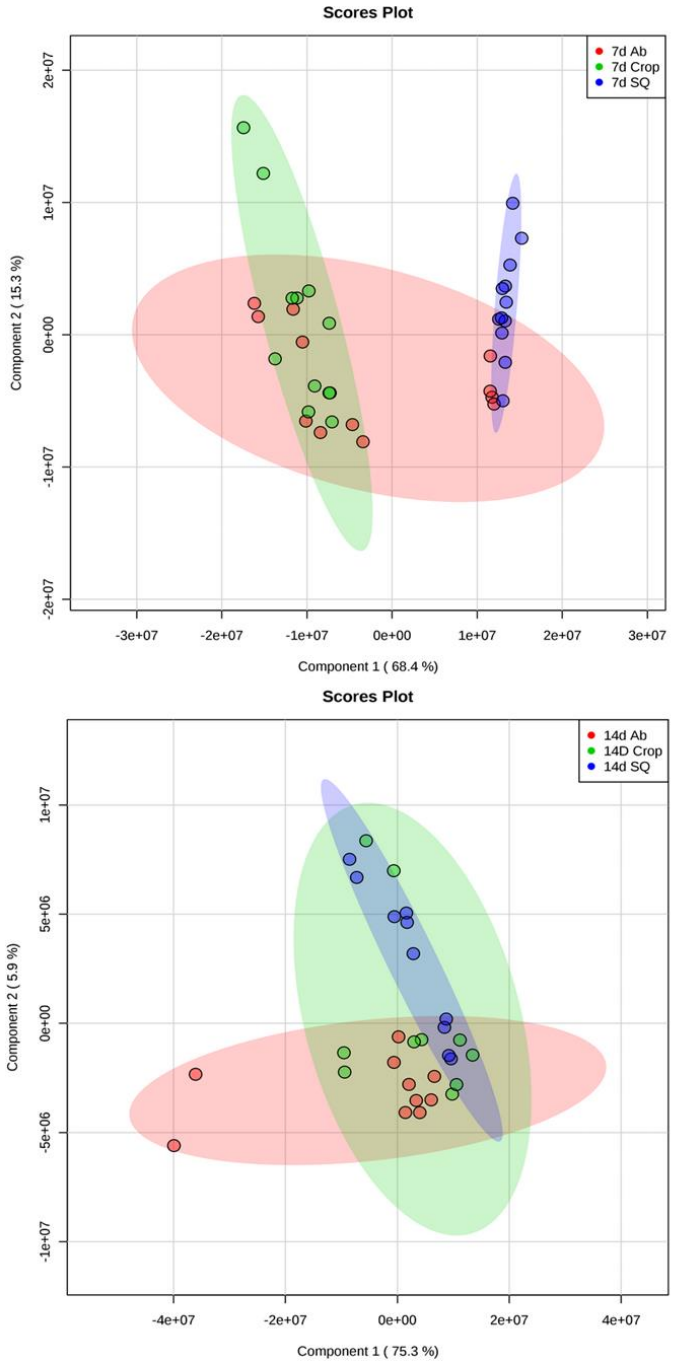

Figure 2.3 PLS-DA visualization of depot differences in elongase and desaturase expression at D7 and D14.

Data from each tissue are encoded by color. Analyses were performed using Metaboanalyst (metaboanalyst.ca) using default settings.

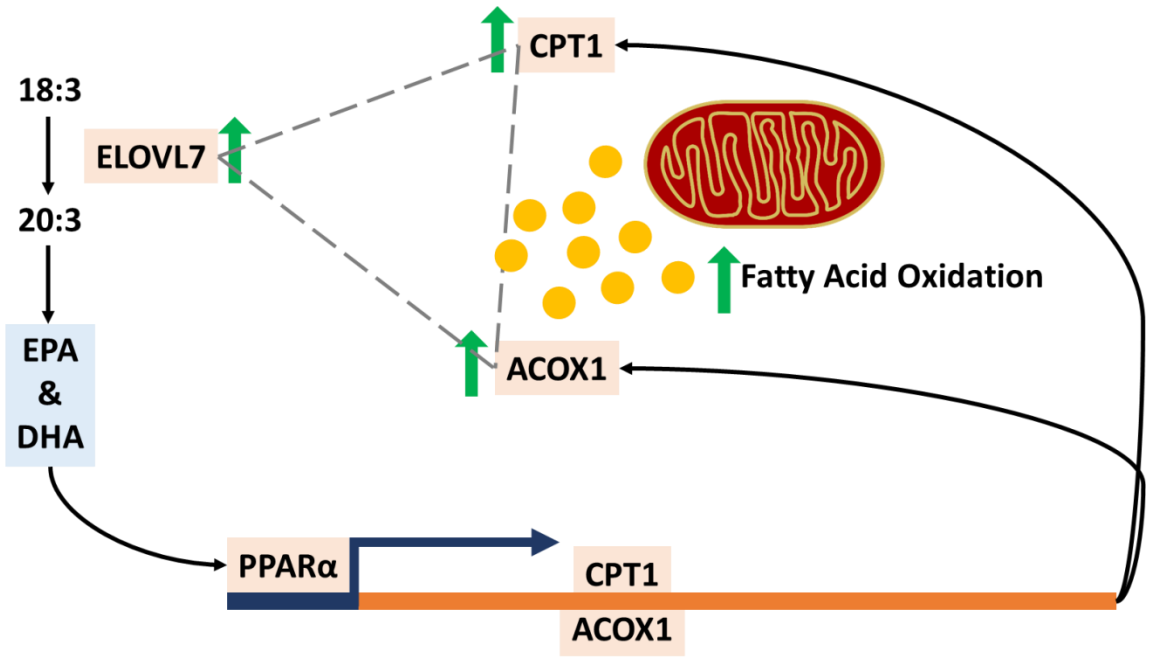

Figure 2.4 Proposed method of increased fatty acid oxidation by upregulation of ELOVL7 and its positive correlation with CPT1 and ACOX1.

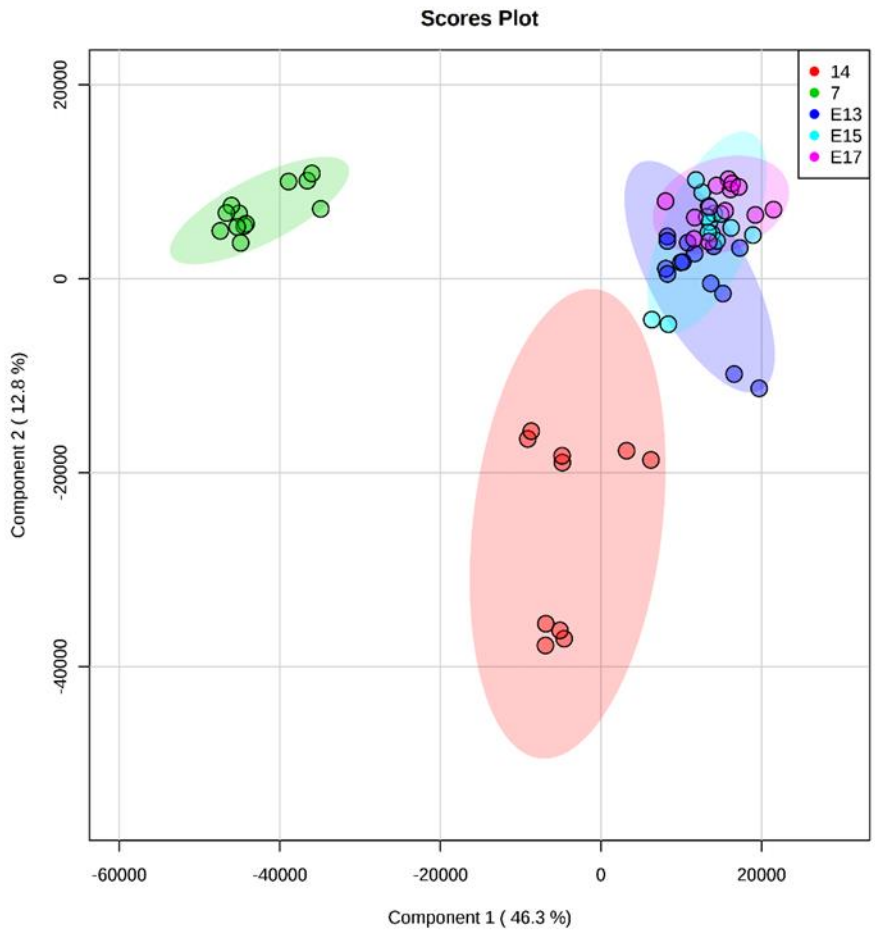

Figure 2.5 Scores plot from PLS-DA visualization of depot differences in elongase and desaturase expression in SQ adipose across embryonic and post hatch development.

Data from each age are encoded by color. Analyses were performed using Metaboanalyst (metaboanalyst.ca) using default settings.

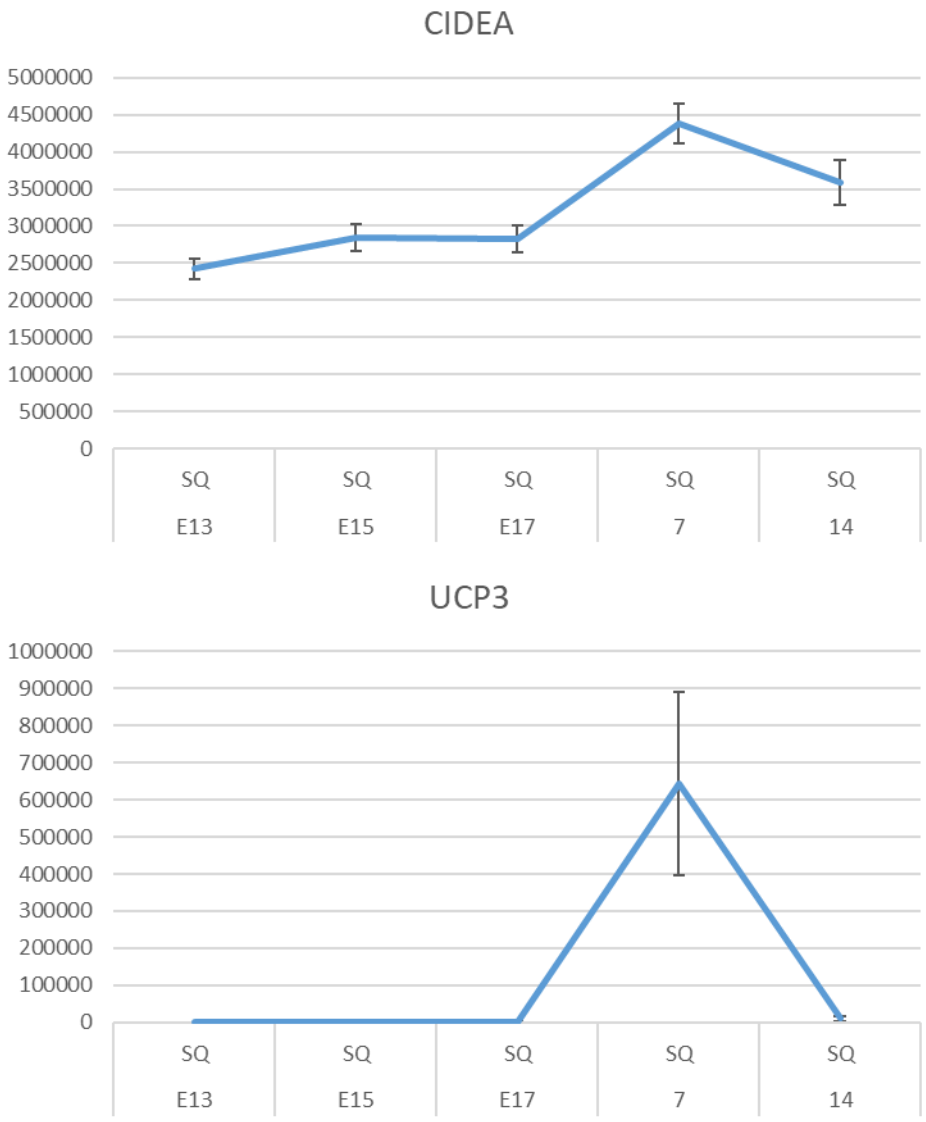

Figure 2.6 Expression of genes involved in adipose tissue browning indicator genes in SQ adipose across embryonic and posthatch development.

Values on the y-axes represent normalized total counts (mean +/- std dev) from targeted RNA sequencing; n=10-14/age. CIDEA, cell inducing death effector a; UCP3, uncoupling protein 3.

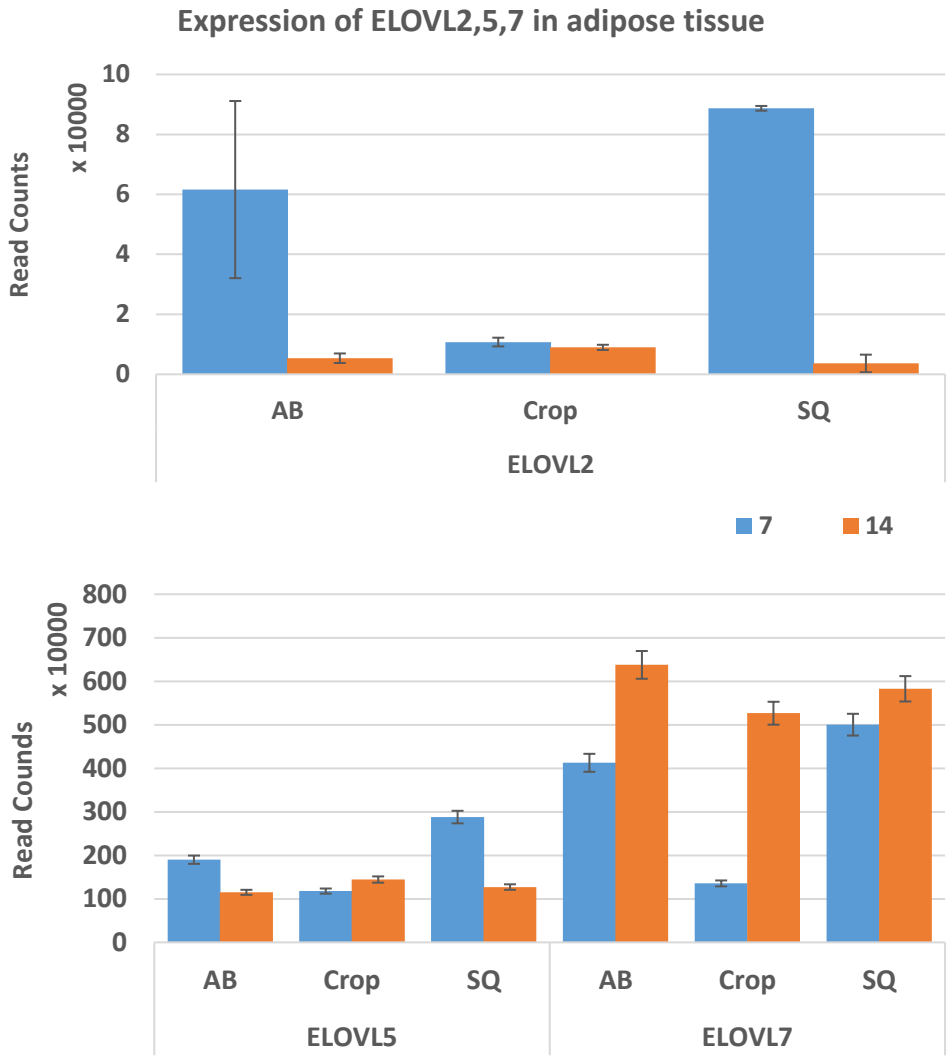

Figure 2.7 Expression of elongases in abdominal (AB), crop and subcutaneous (SQ) adipose depots at post hatch D7 (blue bars) and D14 (orange bars).

Values on the y-axes represent normalized total counts (mean +/- std dev) from targeted RNA sequencing; n=10-12/age. Genes are divided into relatively lower and higher expression to accommodate the range in expression values.

CHAPTER 3
INCREASING CHARCOAL EFFICIENCY FOR BROODING BROILER CHICKENS IN RURAL RWANDA

A version of this chapter was originally published by Robert I. Mihelic, Emily R. Urban, Thomas Gill, Mike O. Smith, and Brynn H. Voy:

Robert I. Mihelic, Emily R. Urban, Thomas Gill, Mike O. Smith, Brynn H. Voy. “Increasing Charcoal Efficiency for Brooding Broiler Chickens in Rural Rwanda.” *African Journal of Poultry Farming* (2019).

Abstract

Rural family poultry production can promote food security and provide income. One challenge facing smallholder poultry producers is the need to brood chicks during the first few weeks of life. In rural Rwandan communities, access to consistent electricity and modern brooding methods is uncommon. Therefore, the traditional method of burning charcoal in clay ovens is used to heat chicks. The rise of charcoal prices in Rwanda has reduced the profitability of poultry rearing. This study examines methods of charcoal reduction for rural small holder (100 bird) poultry farms in Rwanda’s Northern Province. A >50% reduction of charcoal consumption was achieved.

Introduction

Introduction

Rwanda has experienced rapid economic growth and development over the past two decades, but still faces the dual challenges of poverty and malnutrition. Almost 40% of Rwandan households live in poverty, with the majority located in rural areas (NISR, 2015). Limited household income is often associated with limited dietary diversity and a lack of key nutrients, a leading cause of stunted growth and development in children. The Comprehensive Food Security and Vulnerability Analysis report estimates 36.7% of Rwandan children under age five are stunted, based on height for age (MINAGRI & WFP, 2015). Increased access to nutrient dense agricultural products could help alleviate childhood stunting in Rwanda. Options for sustainable intensification of agricultural production to meet the nutrition and income needs of Rwandan households must therefore be investigated (Kayihura & Svirina, 2018; Pritchard, 2013).

Poultry farming is one potential option to increase both family income and the supply of dietary protein in Rwanda (MINAGRI, 2012). Rural Rwandan households often raise poultry, but in limited numbers that make only a modest contribution to family income and nutrition (Mahoro, Muasya, Mbuza, Habimana, & Kahi, 2017). In Rwanda and throughout the region, multiple efforts are underway to teach families how to raise chickens for eggs and meat, addressing both nutritional and economic challenges to well-being (Rockwell, 2016). However, small farmers often lack access to a consistent and affordable source of electricity, which reaches only 12% of rural Rwandans (USAID, 2018b).

Limited access to electricity challenges their ability to raise chicks in larger numbers because of the need for external heat critical for optimal growth and development during the brooding period. Charcoal is typically used in rural areas as a source of heat during brooding (Bello, Adekanmbi, Basiru, & Jagun, 2017); however, charcoal-fueled brooding presents challenges. Charcoal prices for brooding poultry in Rwanda constitute almost one-sixth of the entire cost of poultry rearing (USAID, 2017), and prices are volatile. Charcoal use also conflicts with the Rwandan government's recent regulations against deforestation, which further increases the charcoal price due taxes and regulations and raises environmental concerns (Kanamugire, 2018). Nonetheless, alternative sources of energy for brooding such as natural gas remain cost-prohibitive and are not feasible for near-term efforts to increase poultry rearing by rural farmers (Kanamugire, 2018). Therefore means to optimize the efficiency of charcoal use for brooding, or to identify alternative renewable fuel sources, are critical to increase rural poultry production

Tworore Inkoko, Twunguke

USAID Feed the Future Tworore Inkoko, Twunguke (TI), or "Let's raise chickens and make a profit" in Kinyarwanda, is a three-year pilot project (2016-2019) which aims to increase the capacity of smallholder farmers in Musanze District, Rwanda, to produce broiler chickens. The University of Tennessee Institute of Agriculture (UTIA) implements the activities of TI in partnership with a private feed mill based in Rwanda (Zamura Feeds). Farmers (currently 264) participating in TI raise commercial broiler chicks (~ 100/cycle) from hatch to market age. The coops used are standard across all farmers and are fabricated to be typical of those used in the region. The TI production system was used as a model of rural smallholder farms to test methods to reduce charcoal consumption, and to evaluate the efficiency of biomass as an alternate fuel source. The rationale is that methods which improve fuel use in the TI model should be translatable to other smallholder farmers across Rwanda and the larger region of East Africa.

Materials and methods

Chicks and brooding

Mixed-sex broiler chicks (one day-old) were obtained from a local hatchery in Ruhungeri town and transported to the TI demonstration farm (Musanze District), which is used to train farmers who are enrolled in the TI project. The coops (~ 3.6m x 2.5m) used had concrete floor which were covered with wood shavings as bedding, adjustable plastic curtains to control airflow and temperature, and a corrugated metal roof. Two clay, charcoal burning ovens in each coop were used to brood chicks. Groups of 100 day old chicks were placed into coops outfitted with one of four experimental heating methods:

Charcoal in clay oven (control)

All charcoal was locally produced from eucalyptus wood. Charcoal was burned in two locally made clay ovens per coop. The ovens were approximately 1cm, 45cm and 35cm in dimensions (thickness, height, diameter, respectively). The charcoal brooding method represents the approach typically used for brooding in northern Rwanda.

Mylar hood heat reflector

Two hoods were constructed by shaping polyethylene terephthalate ("mylar") film into a 90cm diameter circle supported by bailing wire (4 mm). A single layer mylar sheet was fasted around the wire frame with tape. A slightly concave shape was created by a small incision made in the center of the hood, a nylon string was passed through the incision, knotted, then reinforced with durable adhesive tape to prevent tearing. The hood was suspended from the rafters 60cm above the clay ovens inside the coop.

Metal hood heat reflector

Corrugated sheet metal, which is commonly used in Rwanda for construction and roofing, was cut into pieces with a surface area of 2463cm2 and suspended above the clay oven inside the coop. Two holes were created 2cm apart in the center and each corner of the hood where a 20cm piece of 4mm bailing wire was passed through each pair of holes and twisted together to create a loop. Nylon string was tied around each loop then knotted 30cm above the center of the hood to give it a slightly concave shape. Each hood was suspended 30cm above a clay oven by fastening the nylon string to the coop rafters.

Biomass briquettes

Manufacturing of biomass briquettes was performed by Habona Ltd at the Kitabi Eco-center in Rwanda's Southern province and purchased for 200RWF/Kg. Briquettes were formed by compressing and charring saw dust (60%) and rice hulls (40%) and then boring a 1cm hole through the center. Briquettes were tested in conjunction with the metal hood heat reflector and used as a charcoal substitute. Briquettes were burned in a similar way as charcoal by placing them in the clay ovens and lighting with matches. Once lit the biomass burned completely without agitation or being relit.

Experimental brooding was performed for two weeks, the same duration used by TI farmers. For each brooding method average coop temperatures ranged between 29^{o}C and 35^{o}C. The amount of charcoal needed to maintain optimal temperature range during brooding was weighed to assess fuel use.

Chick performance in each group was assessed based on weight gain. Thirty-three chicks from each treatment group were randomly selected and weighed at placement (day 0) and at one, two and three weeks of age to obtain weekly growth rates.

Heating efficiency

Heat production during a defined burn period was measured to quantify efficiency of charcoal and biomass briquettes. Five clay ovens for each treatment were loaded with 2kg of either charcoal or biomass briquettes. A third set of ovens was used to evaluate the benefit of adding volcanic rocks to burning charcoal, due to anecdotal reports that farmers add volcanic rocks to increase heat retention. For the charcoal with rocks method, five volcanic rocks weighing a total of 0.5kg were mixed with burning charcoal 60 minutes after lighting. After lighting the fuel source, temperature was taken on four equally-spaced sides of each oven every 60 minutes using a Lazergrip 1018 Inferred Thermometer (Etekcity; Anaheim, CA). The four measures at each time point were then averaged, giving one temperature for each oven per hour. Both charcoal and biomass were lit once and allowed to burn without agitation until combustion ceased.

Statistics

Effects of brooding treatments on chick growth and heat generation were analyzed statistically using SAS (version 9.4; Cary, NC), with significant effects of treatment defined by p-values < 0.05. Effects of the different brooding treatments on chick growth were analyzed using repeated measures mean separation after normalizing data by square root transformation. Area under the curve (AUC) values for heat generation were compared using ANOVA.

Results and discussion

Many programs that use poultry rearing as a means of poverty alleviation have arisen in the past 20-30 years (Glatz & Pym, 2013). Currently, although there are concerns about its future costs and environmental sustainability, charcoal is the most readily-available fuel small holder farmers can use for brooding poultry. Therefore means to reduce the amount of charcoal would be of immediate benefit to farmers. Adding either a mylar or metal hood to the control (charcoal in clay oven) brooding method reduced charcoal consumption considerably. The control, mylar hood, and metal hood coops used 55.4, 29.7, and 25.3 kg of charcoal, respectively (Figure 3.1). The ability of the two experimental manipulations (mylar and metal hoods) to effectively support brooding was reflected in chick growth. There were no significant effects of either experimental brooding method (mylar hood or metal hood) on final chick weights ($P=0.22$) or on the total weight gain at three weeks of age ($P =0.21$;Figure 3.1). Therefore, each of the two hood types maintain growth, the primary criterion for successful brooding, despite considerable reductions in charcoal use.

Smallholder poultry producers in the TI program currently use an average of three bags of charcoal during the approximately two-week brooding cycle (USAID, 2018a). The reduction in charcoal need provided by either the mylar or metal hood therefore would be expected to save farmers approximately 18,000

RWF per growing cycle, based on the current average price of charcoal (~ 12,000 RWF/bag; personal communication). Based on the current average TI farmer income of 50,000 RWF/cycle, the installation of a hood would result in a 36% increase in farmer profit (TI, unpublished data). With rising costs for charcoal (costs have increased by 36% in the past 24 months (Kanamugire, 2018; Niyitegeka, 2016)) actualized savings could be even more than the initial estimate. While the mylar hood reduced charcoal use to approximately the same extent as a metal hood, the mylar was prone to rapid degradation and may fail if used for multiple brooding cycles. Nonetheless, mylar may be a viable option in some cases due to its minimal cost (approximately 220RWF/m2; (Amazon.com, 2018)), light weight, and ease of transport. The metal hood had a much longer life span, as the same hoods were used in multiple cycles with minimal deterioration. Removing dust and ash off the hood between brooding cycles and placing the hood at least one foot above the oven could have positively contributed to longevity.

As a follow-on experiment, the ability to further reduce charcoal use by temporarily dividing the coop area (half-house brooding) was evaluated in conjunction with the control charcoal oven method. The half house brooding approach is used commercially in intensive production systems (Overhults, 2017). A heat resistant plastic curtain (the same material used in coop construction) was suspended from the ceiling and hung to the floor, effectively dividing the coop into two halves, with one half used for brooding. Only one clay oven was used to brood chicks in the half-house system. Charcoal consumption was only measured in bags of charcoal. When combined with use of the metal hood, functionally dividing the coop in half reduced charcoal by an additional half of a bag. Over the two week brooding period, the combination method would be expected to reduce the amount of charcoal required by another half-bag of charcoal, equivalent to 6,000 RWF (projected 12% increase in profit) compared to the current charcoal/clay oven method alone. Segmenting any brooding facility is easily accomplished by installing a heat resistant fabric or plastic around the heating source. The material used should be cost-effective, reusable for multiple brooding cycles, and not deform when exposed to heat.

Alternative fuels derived from various types of biomass are under development in Rwanda and other countries (Lamido, Lawal, & Salami, 2018). The biomass briquette tested here was created from sawdust and rice hulls. The experimental coop fueled with briquettes used 41.6 kg of biomass the first week, but was then terminated due to excessive smoke production. If brooding had continued, biomass brooding would require ~ 83 kg for two weeks, which is approximately 33% more than traditional charcoal brooding, and ~ 65% more than using a mylar or metal hood. While the biomass briquettes tested in this study were deemed unsuitable for brooding, different biomass formulations or sizes could produce alternative results as individual components tend to contain varying levels of combustible energy. For example, rice hulls and sawdust, components of the biomass used, contain less combustible energy (13.5 and

18.1 MJ/kg, respectively) than eucalyptus (19.3 MJ/kg), which is more commonly used to fabricate charcoal in Rwanda (Saidur, Abdelaziz, Demirbas, Hossain, & Mekhilef, 2011). The heating potential of other alternative types of biomass should be explored in further studies. A number of internal and external organizations in Rwanda are developing innovative cooking and heating methods (Jean de Dieu & Kim, 2016), some of which could potentially be appropriately modified to brood chickens.

Anecdotal interviews conducted as part of the TI project revealed farmers often add volcanic rock to charcoal fires to optimize heat production. The impact of adding rocks was quantitated by measuring heat generation across an 11 hour burn period in charcoal fires with and without added volcanic rock. Heat production from fire fueled by biomass briquettes was also measured to quantify efficiency of the biomass heat source (Figure 3.2). Charcoal, charcoal with rocks, and biomass briquettes produced peak temperatures of 280.8^{o}C, 257.1^{o}C, and 197.6oC retrospectively. Total heat generated, reflected by area under the curve (AUC), differed significantly across the fuel sources (P=0.017). As expected from the lower peak temperature, the biomass briquettes generated less heat (2541 ± 287) than either of the charcoal-fueled fires (3294 ± 364 and 3663 ± 175 for charcoal and charcoal with rocks, respectively). Adding rocks to the charcoal increased the amount of heat produced over the 11 hours by approximately 11%, but the increase was not statistically significant (P= 0.18).

High input small holder broiler farming requires brooding methods other than natural hen brooding. Little literature is available concerning brooding broiler chickens in the absence of electricity for village poultry. The majority of low input famers rely on natural hen brooding as opposed to an exogenous heat source (Mahoro et al., 2017), likely due to a small number of household birds (<15) which are commonly raised by brooding hens (Glatz & Pym, 2013). Hen brooding method is not feasible for more intensive operations in Rwanda which raise up to 500 chickens at a time (Mbuza, Manishimwe, Mahoro, Simbankabo, & Nishimwe, 2017). Three month survival rates for hen brooding have been reported to be as low as 40% when weather is cold or rainy. (Demeke, 2012). In areas where electricity is inconsistent either charcoal or hay-box brooders are used for more intensive operations (Habte et al., 2017). The effectiveness of hay-box brooders is also highly dependent on weather. In seasons of heavy rains in Ethiopia survival rates of 60% have been reported. Chicks brooded in a hay-box report slower growth rates during 4-6 weeks making hay-box brooding an unsuitable method for brooding broilers which are typically harvested at 6 weeks old (Demeke, 2012). If access to consistent electricity is available conventional brooding methods such as heat lamps or canopy brooders are generally recommended. Although charcoal is not ideal for small holder intensification, it is currently the most feasible method for the majority of farmers in regions of Africa similar to Rwanda where access to electricity is inconsistent or unaffordable.

In conclusion, the metal hood and half house brooding methods, which are similar to techniques used in other countries with strong conventional poultry

sectors, have the potential to be implemented throughout Rwanda in poultry facilities of any scale. While efficiencies and burn rates are likely to differ according to coop size and environmental conditions, the approaches presented in this study could reduce energy consumption for brooding chickens in many different areas without access to consistent electricity.

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I express my gratitude to Drs. Brynn Voy, Jennie Ivey, and Lannette Edwards, Mike Smith, Thomas Gill, and Emily Urban for their valuable input and comments. This manuscript is made possible by the generous support of the American people through the United States Agency for International Development (USAID). The contents are the responsibility of The University of Tennessee and do not necessarily reflect the views of USAID or the United States Government. This study was conducted with the financial support of USAID/Rwanda and the African Sustainable Agriculture Project (ASAP) Foundation as a Global Development Alliance, Tworore Inkoko, Twunguke (Let's raise chickens for profit). The research was led by the University of Tennessee Institute of Agriculture (USAID Cooperative Agreement No.: AID-696-A-17-00006) with subaward to Zamura Feeds Limited, Rwanda. I also thank The University of Tennessee Animal Science Department for education and funding.

Appendix

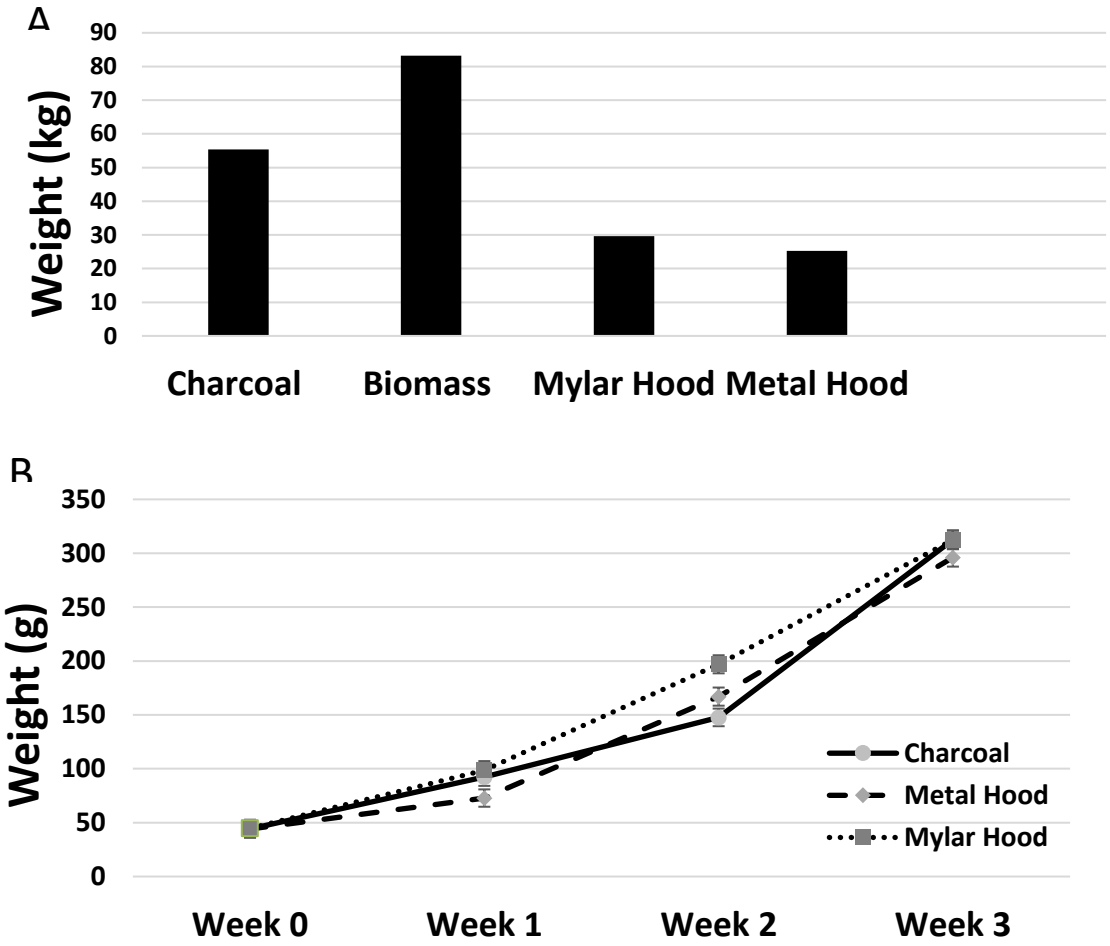

Figure 3.1 Effects of different brooding methods on fuel usage.

(A) and chick growth (B). (A) Fuel use (g/brooding cycle) of four different methods of heating smallholder broiler coops at the TI demonstration farm, Musanze district, Rwanda; (B) Effects of each brooding method on chick growth, based on body weights at placement (Week 0) and after one, two and three weeks of brooding, n=27-33/method. Chicks in the biomass briquette group are excluded due to early termination because of excessive smoke production. Each data point represents average chick weight +/- standard error. Initial weights (week 0), final weights (week 3) and weight gain did not differ significantly between groups ($p>0.05$).

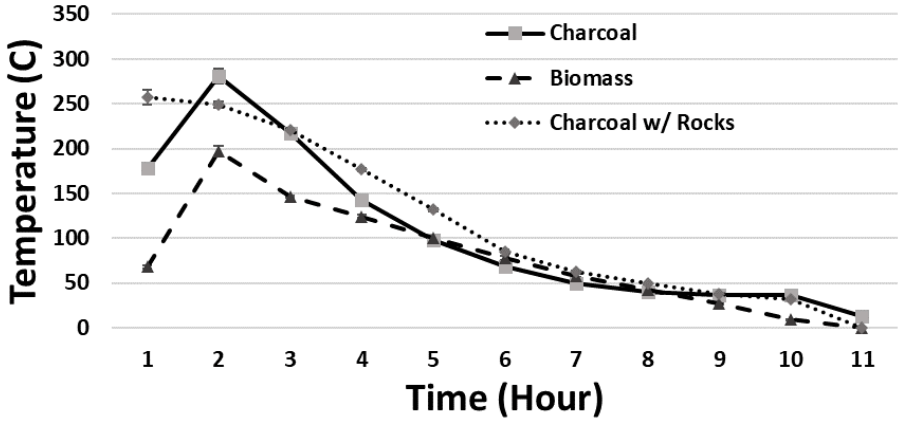

Figure 3.2 Heat production.

Heat Production from ovens fueled by charcoal (control), charcoal with addition of volcanic rocks, and biomass briquettes. Equal masses of charcoal or biomass briquettes were added to each oven (n=5/method) and temperatures were measured hourly across 11 hrs. Each data point represents average temperature ± standard error. Area under the curve (AUC) was calculated for each method and compared using ANOVA.

Figure 3.3 Charcoal ovens used to brood chicks.

Two clay ovens were used in all treatments except the half house method where only one oven was used.

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CHAPTER 4 CONCLUSION

As global populations continue to rise the need for efficiently produced, sustainable protein is vital to ensure proper food security for future generations. The poultry industry has rapidly grown since the 1950's due to its continual efforts to increase efficiency. The studies presented examined ways to increase poultry efficiency and sustainability in the United States and in rural Rwanda. Utilizing targeted RNA sequencing the first study found a change in expression profiles of enzymes responsible for synthesizing eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), two fatty acids associated with fat reduction for chicks -8, -6, -4, +7, and +14 days from hatch. Many genes showed significant expression changes at D7 which likely correlates to the extreme metabolic shift chicks go through at hatch. The gene ELOVL7 might be responsible for the synthesis of EPA and DHA in young broilers. In Rwanda a half house brooding method increased charcoal efficiency for brooding >50% for small holder farmers involved in the TI project. The charcoal reduction could result in increasing environmental sustainability by reducing deforestation for charcoal production. Farmer income is projected to increase by ~$100/year as a result of reduced charcoal consumption. When the TI project is at full capacity (750 farmers) savings are projected to $75,000, at current charcoal prices, for the TI project. The further increase in efficiency in Rwanda will enable the poultry industry to expand into more rural areas potentially leading to an increase in protein consumption for rural Rwandans. The half house brooding method could be implemented in small holder farms across sub-Saharan Africa and other areas with limited access to electricity to more efficiently grow broiler chickens.

VITA

Robert I. Mihelic was born on October 12, 1994 in Gadsden, Alabama. He was raised most of his life in Knoxville, Tennessee by his parents Matt and Rebecca Mihelic with his older siblings Melinda, Matthew, and Michael. He graduated high school from the Christian Academy of Knoxvile and received an Associate's of Science from Pellissippi State. While attending Pellissippi State Rob gained an interest in biology and animal science. Rob then pursued a Bachelor's of Science in Animal Science from the University of Tennessee. As an undergraduate he became interested in molecular biology and began to work as an undergraduate researcher in the Dr. Brynn Voy lab group. Rob was invited to stay on at the University of Tennessee as the first 5 Year BS-MS student in the Department of Animal Science under the mentorship of Dr. Brynn Voy.