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Improving body composition in broiler chicks through the early life diet

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I am submitting herewith a thesis written by Kamille Piacquadio entitled "Improving body composition in broiler chicks through the early life diet." 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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Improving body composition in broiler chicks through the early life diet

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ABSTRACT

The poultry industry relies heavily on the efficient growth performance of broilers to provide quality breast meat at a low cost to meet consumer demand. However, high efficiency of broilers is also related to the occurrence and severity of skeletal muscle abnormalities like wooden breast. This study investigated the efficacy of incorporating long-chain omega-3 polyunsaturated fatty acids, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), into the diets of hens and their offspring on altering the causative features of wooden breast. Early exposure to these fatty acids in broilers has shown to reduce adiposity, and may address excess adipogenesis seen in wooden breast. Physical characteristics of weight gain and relative breast weight were measured to confirm that fish oil did not compromise growth. Histological analysis of the breast showed that the perinatal fish oil diet promoted growth of larger muscle fibers and the maternal fish oil programmed reduced adipocyte size. Gene expression analysis results proposed the mechanisms that prompted differences in muscle fiber size area between diets namely, the increase in early myogenic marker expression, *PAX7* and *MYOD1* and an increase in a marker of myogenesis and angiogenesis, *PDGFRβ*. The increased expression of *FABP4* seen in the perinatal fish oil diet groups may be attributed to a relatively new understanding of its function in muscle, and potentially an increase in fatty acid oxidation. Altering the hypertrophic growth rate of high efficiency broilers may assist in preventing growth that exceeds nutrient and oxygen provision seen in wooden breast. Primary satellite cells were collected from a subset of chicks that only differed by the maternal fish or soybean oil diets. Satellite cells isolated from the maternal fish oil group accumulated significantly less triglyceride versus maternal soybean oil. Decreasing elevated adipogenesis seen in the

development of wooden breast may help to lessen the severity of the myopathy. In conclusion, future research is needed to identify the long term impacts fish oil supplementation can have on muscle development and adiposity in broilers at market age.

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INTRODUCTION

The poultry industry and creating the modern broiler

The poultry industry in the United States in 2018 provided approximately 1,393,739 total jobs, and yielded $347.08 billion in total economic activity, and continues to grow nationally (1). Consumption over the course of the past 60 years has grown from 23.6 pounds in 1960 to a forecasted 94.4 pounds per capita in the year 2020 (2). In comparison to other meat protein available on the consumer market in 2019, chicken protein was estimated to be $4 less per pound than other leading meat proteins available. If the average person consumed the same amount of other available meat products as chicken protein was consumed in 2019, the consumer would pay almost $400 more per year (3). In addition to affordability, chicken is also a rich source for complete protein. The recommended general intake to maintain adequate muscle protein synthesis over a 24-hour period in younger and older adults is two to three meals per day that contain 25 to 30 grams of protein (4). The USDA National Nutrient Database states that a skinless, boneless cooked or roasted chicken breast contains 31 grams of protein, sufficient for a meals' worth of protein requirements. In an effort to satiate demand, approximately 90 billion broiler chickens are now produced in the US each year.

Selection for rapid growth has allowed supply to meet demand. Transformative steps in the poultry community were taken after the conclusion of the Second World War. Demand for chicken protein soared, and alongside the increasing availability of beef and pork, the poultry market needed to keep up. The "Chicken of Tomorrow" contest, which was sponsored by the A&P Grocery Company, fostered the development of selective

breeding programs that led to modern broiler lines in use today (5). Developers at the time were faced with the task of selecting traits of broilers that were easily quantifiable and very heritable: body weight, feed consumption, feed conversion ratio, and yield. Consequently, broilers in 2005 at day 56 weighed 4,202 grams compared to their nonselected counterparts in 1957 at day 56 weighed 905 grams (6). Modern broilers reach market age at approximately 47 days, weighing an average of 2.9 kg. In contrast, broilers in 1955, as selection programs began to develop, required 70 days to reach a weight of approximately 1.4 kg. Moreover, modern broilers need 50% less feed to support this growth than their unselected predecessors (7).

Multimorbidites associated with selection for rapid growth

Inadvertent consequences have arisen from intense selection for rapid growth. For example, modern broilers deposit excess adipose tissue compared to unselected lines. The increase in fat accretion is concerning as it wastes feed, the most costly component of production, skews consumer perception of product, and increases unwanted byproduct therefore, increasing labor cost for trimming the fat (8). Susceptibility to ascites has also increased because the heart and vascular system do not develop in proportion to the metabolic demand of rapidly growing breast muscle. Ascites is the leaking of fluid into the peritoneal space and pericardium from the increase in venous pressure due to volume overload and right ventricular failure (9).

Breast muscle myopathies (BMM) are a family of disorders that also arose as a consequence of rapid growth. The myopathies are the occurrence of spontaneous muscle abnormalities that are affiliated with the cellular dysfunction and metabolic imbalance

triggered by the energy demand from the rapid rate of muscle growth. Breast muscle myopathies are not apparent until post-mortem processing, because there are no clear changes in growth or health status of affected broilers. Affected breast muscle can have either dispersed or localized changes in meat quality, and currently there are no pathogens or infections associated with BMM. A few well-defined myopathies include deep pectoral myopathy (DPM), wooden breast (WB), and white stripping (WS); (10). These myopathies are very costly to the industry because they compromise the quality of the most valuable meat component of the bird. The two most common myopathies are white striping and wooden breast. White striping is characterized by fibrotic striations that are white in color and run in parallel to muscle fibers. Wooden breast is named because it causes hardening of the breast filet. Affected muscle becomes fibrous and tough, and must be used for lower profit products (e.g., processed chicken nuggets) rather than sold as breast filets (11). The incidence of these disorders has increased sharply in recent years; up to 90% of flocks of the most efficient broiler lines are estimated to be affected (12). According to Kuttappan et al. (13), production of broiler live weight in 2016 was 53 million pounds, which is approximately 12 million pounds of breast meat, suggesting a conservative estimate of $200 million per year lost due to both decreased yield and devalued product. More recently, estimates have grown to almost $1 billion economic losses per year (12). Consumers' perception of breast meat that is afflicted with a myopathy considers the breast meat to be less desirable because of their color, texture, and understand them to be more "fatty" due to stripping, in addition to decreased protein content. Importantly, neither disorder is restricted to a specific genetic line. Rather, severity is directly related to growth rate, and

lesions are only found in breast muscles, which grow out of proportion to other muscles in broilers (10). These facts suggest that conditions may instead result from a metabolic imbalance that is secondary to the demands of rapid growth.

Wooden breast syndrome etiology

Wooden breast is classified as the hardening of the pectoralis major (*p. major*) muscle, most commonly found on the proximal end of the muscle, and in severe cases, the hardening can be distributed throughout the filet. Studies have suggested that the selection for rapid growth in both body weight and breast muscle weight is closely affiliated with WB affected broilers. Conformational reports revealed that broilers who observe expedited weight gain from early stages (1 and 2 weeks of age) have a higher potential to develop WB upon reaching market age (14). The most informative studies of causative changes in breast muscle myopathies have been performed with wooden breast. The histological characteristics of wooden breast reveal the infiltration of macrophages, degeneration and regeneration of muscle fibers, and increased growth of adipose and connective tissue (15).

A study conducted by Papah et al. (14) utilized birds from a high-breast-muscle-yield purebred broiler line in pursuits to identify early pathologies of WB. Across all time points measured, lipogranulomas, inflammatory cells that surround lipid droplets associated with fibrosis, were found between vessels and myofibers in the majority of broilers. Similarly, macrophages were found to have infiltrated venous tissue in as early as 1 week of life. Additionally, ectopic lipid accumulation in the extra cellular matrix (ECM) of myofibers was detected at week 4 of age as an early lesion of WBD. Displays of ectopic lipid accumulation coupled with lipogranulomas, and macrophage infiltration contribute to

the consequent fibrosis, and development of the muscle hardening myopathy (13). Gene expression analysis in affected breast muscle of market age birds showed an increase in genes associated with inflammation, fibrosis, and lipid metabolism (16, 17). The association between lipid metabolism dysregulation and the development of wooden breast was further confirmed by metabolomics data. Reports from the metabolomics analysis of wooden-breast affected birds (18) showed increased levels of lipid catabolism products including glycerol, in concert with findings of high levels of metabolites that reflect activity of vitamin B6, a vital coenzyme in lipid metabolism (19). Gene expression analysis also revealed increased expression of lipid metabolism genes at two weeks of age in chicks that eventually developed wooden breast (17). Included in this set of genes is *PPARγ*, a master regulator of adipogenesis. Downstream targets of *PPARγ* include genes that promote fatty acid uptake, and several of these genes including *LPL* and *FABP4* were also upregulated in wooden breast vs. healthy breast samples. These results suggest that the early pathology of the disease is associated with metabolic dysregulation, and that ectopic adipogenesis within breast muscle may contribute to these myopathies.

Intramuscular fat and satellite cell physiology

The quality of meat products is associated with desirable traits such as juiciness and tenderness, influenced by intramuscular fat (IMF), muscle fiber type and growth rate. Intramuscular fat is not visible or able to be anatomically differentiated in chicken, creating difficulty in identifying the mechanism of IMF deposition (20). The cellular population responsible for IMF accumulation are mature adipocytes that contain large lipid droplets. The adipocytes are located between muscle fibers in a muscle fiber bundle as well as found

between fiber bundles (21). Mesenchymal stem cells (MSCs) have been identified to be present in multiple adult tissue types such as skeletal muscle, and are responsible for adipocyte, chondrocyte, osteocyte, and myocyte populations. Myocytes, the differentiated muscle cell, originate from MSCs, but are formed from sequential steps involving cell determination and differentiation. Satellite cells (SCs) are responsible for differentiating into myocytes which are cells that fuse to formulate myotubes.

Satellite cells, also called adult myoblasts, are an undifferentiated stem cell population that contributes to hypertrophic muscle growth during development and to muscle regeneration in adults. They are named "satellite" cells because they reside in the perimeter of muscle fibers. Satellite cells contribute to hypertrophic growth by differentiating into myoblasts and fusing to form myofibers or by fusing with existing myofibers. The phases from progenitor cell to myotubes are symmetrical during embryonic development and adult myogenesis. During the specification phase from progenitor to satellite stem cell, high levels of *PAX3* are expressed. *PAX3* is responsible for recruitment of progenitor cells, and tapers off in expression as satellite stem cells have become identified. The satellite stem cell remains to be malleable in cell commitment until expression of *PAX7* heightens to establish a subpopulation of satellite stem cells as a self-renewing population or promotes commitment to satellite cells from progenitor stem cells. *PAX7* expression is imperative to continue to renew the pool of quiescent satellite cells or allow for regeneration of muscle fibers, emphasizing *PAX7* as a main defining factor for this cell type (22). Once satellite cells have become committed, their activation and proliferation into myoblasts occurs, driven by the expression of *MYOD1*. *MYOD1* is

expressed through early differentiation, in which myoblasts become myocytes. However, *MYOD1* expression begins to decrease in tandem with *MYOG* expression's increase, as myocytes begin to fuse with other myocytes, forming multinucleated muscle cells, known as myotubes (23). Although the primary role of satellite cells is as precursors for myoblasts, they are multipotent stem cells that also have the ability to differentiate to bone and to adipocytes (24).

Satellite cells are not a fixed population of cells, in fact their heterogeneity is specific to their muscle origin. In *p.major* specifically, SCs have been shown to be more susceptible to change their terminal differentiation fate from myocytes to other cells types such as adipocytes in the presence of nutrient or thermal stimuli (25, 26). In chicks, SCs proliferate during the late stages of embryogenesis and up until approximately two weeks after hatch, when they become quiescent (27). Their plasticity is thought to be at its highest during late embryogenesis and just after hatch, when their rates of proliferation and differentiation are very high (28). Relatively little is known about regulation of SC adipogenesis *in vivo*. The availability of food post-hatch and varying the levels of methionine in the diet have been shown to influence adipogenesis, which demonstrates that diet may be a factor in changing terminal satellite cell myogenic differentiation (29). Therefore, there may be potential for the lipid accumulation that is presently seen in WB affected broilers to be regulated by the change in commitment of satellite cells from myogenic to an adipogenic cell lineage, in addition to the population of adipocytes that produce lipid in the breast muscle.

Polyunsaturated fatty acids

Long-chain polyunsaturated fatty acids (LC n-3 PUFAs) may be a tool with which to reduce ectopic lipid accumulation in breast muscle and limit the incidence or severity of wooden breast. This class of fatty acids shares the feature of having the first double bond located at the omega-3 carbon in the fatty acid chain. Similarly, omega-6 PUFAs have their first double bond at the omega-6 carbon. Notable long chain omega-6 PUFAs include: linoleic acid (LA) (18:2 n-6), gamma linoleic acid (GLA) (18:3 n-6), and arachidonic acid (AA) (20:4 n-6). In addition, long chain omega-3 PUFAs worth recognizing include: alpha-linolenic acid (ALA) (18:3 n-3), eicosapentaenoic acid (EPA) (20:5 n-3), and docosahexaenoic acid (DHA) (22:6 n-3). In humans and other organisms the endogenous synthesis of LC n-3 and n-6 PUFAs including and exceeding 18 carbon chains is impaired. Therefore, all sources of LA and ALA are exogenous and must be obtained through the diet.

The n-3 PUFAs are direct competitors to n-6 PUFAs for activity in cellular membrane formation, provision of energy, and formation of eicosanoids. Eicosanoids are signaling molecules that are often affiliated with inflammation and vasoconstriction if derived from an n-6 PUFA, opposite from n-3 PUFA impacts in most cases (30). In addition, competition between n-6 and n-3 fatty acids in which ratios of n-6 are higher tend to decrease and replace LC n-3 PUFA in developing tissues (31). Foods that are rich in n-6 PUFA include animal fats and vegetable oils, such as corn oil which also contain considerable levels of saturated fat. Foods that contain high levels of n-3 are flaxseed, walnuts, and fresh water fish which are especially high in EPA and DHA (32). Kalakuntla et al. (33) investigated whether incorporating various n-3 PUFAs in broiler chicken diets

in the starter and finisher phases would impact overall quality and additional sensory attributes while also effectively enriching the meat in n-3 PUFA. Enriching animal products in n-3 PUFA is of growing interest since consumption of n-3 PUFA is linked to multiple health benefits in humans. Kalakuntla et al. (33) reported that enrichment of the starter and finisher diets significantly increased the n-3 PUFA content, while lowering both n-6 PUFA deposition and the n-6:n-3 ratio in breast and thigh without impacting overall performance.

Dietary LC n-3 PUFAs, EPA and DHA that are found in large abundances in marine oils, have been shown to suppress adipocyte differentiation in multiple species. A study done in broiler chicks enriched the starter diet with four different types of fat, including fish oil and fed these diets from 7 to 30 days of age. Data showed that fish oil supplementation yielded significantly smaller adipocyte volume and increased concentration of plasma NEFA, suggesting a promotion in fatty acid mobilization to peripheral tissues, limiting adipocyte lipid accumulation (34).

Developmental programming through dietary enrichment

Critical changes to the environment during embryogenesis or immediately following birth or hatch has been shown to have long-term effects on an organism's physiology. In fact, studies have shown the diet of the mother can have a programming effect on the offspring. Kehler et al. (35) highlighted the impacts of the maternal diet in mice by supplementing dam diets with high fat or low fat chow. The offspring of these dams were all sired by males fed a low fat diet, and provided milk immediately after birth by a low fat chow fed female. The offspring were then divided further by being fed chow

high or low in fat creating a 2x2 matrix after weaning until 17 weeks of age. While the diet after birth impacted the offspring on a greater scale, the maternal high fat diet had a programming effect that extended throughout adulthood.

The developing chick embryo is an effective model for measuring the effects of a maternal diet enriched in LC n-3 PUFA. The components of the hen's diet are directly incorporated into the yolk that the chick embryo uses for growth and development over the course of 21 days until hatch (36). A study done in broiler chicks utilized LC n-3 PUFA supplementation in the embryonic period by feeding Cobb500 broiler-breeder hens a diet supplemented with fish oil for 28 days and subjected the offspring from both maternal groups to the same commercial starter diet post-hatch. Results showed that supplementing the prenatal environment in EPA and DHA had a programming effect that reduced adiposity by approximately 38% in both abdominal and subcutaneous depots at 14 days post hatch. Additionally, adipocytes at day 14 in chicks from a fish oil maternal diet were smaller in volume, but greater in number. The structural composition of lipid droplets in adipocytes can be suggestive of lipid metabolism activity. Beckford et al. (37) suggested that the size of adipocyte paired with increased gene expression of proteins responsible for fatty acid mobilization, may indicate an increased activity of lipid utilization in adipocytes.

Enhancing broiler chicken body composition through diet

The use of selection for rapid growth in modern broilers has resulted in unforeseen consequences such as an increase in abdominal adipose tissue, and breast muscle myopathies. In pursing solutions for these side-effects, researchers have turned to methods such as feed restriction and further manipulation of commercial diet components. Previous

studies have been successful in manipulating broiler abdominal fat pad weight and phenotype with the use of LC n-3 PUFA supplementation in the maternal and post-hatch diets respectively (34, 37). The LC n-3 PUFAs have been shown to act as signaling molecules during the late stages of adipocyte differentiation, and suppress gene expression of regulators that promote differentiation of pre-adipocytes to adipocytes in rats (38). Growing evidence suggests that LC n-3 PUFA supplementation is advantageous to suppress lipid accumulation in 3T3-L1 adipocytes both by limitation of fatty acid synthesis and triglyceride production, and repress adipocyte differentiation (39).

In addition to attenuating increased adipose growth, EPA and DHA supplementation have been shown to benefit muscle development. Supplementation of EPA and DHA have shown to mitigate the negative effects of inflammation on skeletal muscle differentiation, and satellite cell function. Tumor necrosis factor-α (TNF-α), a cellular mediator in response to injury, has shown to suppress myogenic differentiation and promote apoptosis of myotubes, resulting in muscle loss. A study conducted in C2C12 cells were differentiated in the presence or absence of TNF-α with EPA as a co-treatment, pretreatment or without EPA. Results indicated that EPA in both the pre-treatment and co-treatments prevented the inhibitory effects TNF-α has on myotube differentiation (40). In a follow-up study, EPA treatment was able to decrease nuclear factor-kappaB (NF-κB) expression, a cellular mediator affiliated with impairment of skeletal muscle differentiation, and an increase in peroxisome proliferator-activated receptor gamma (PPARγ) expression (41). A specific member of the PPARγ coactivator family and crucial regulator of energy metabolism, PGC-1α, has been studied for a potential association with

myogenic differentiation. Studies have shown that PGC-1α was upregulated during myoblast differentiation (42). These findings may suggest that there is a reciprocal relationship between inflammatory cell mediator expression and expression of PPAR related genes in relation to inhibited or promoted myogenic differentiation, respectively. The LC n-3 PUFAs may also enhance myogenic differentiation via these pathways by suppressing inflammation and upregulating expression of genes promoting myogenic differentiation. Another integral part of building muscle mass is appropriate protein synthesis. Eicosapentaenoic and docosahexaenoic acid supplementation was used to assess protein synthesis changes and explore their potential role in insulin-mediated protein metabolism. Gringas et al. (43) supplemented the diet of growing steers with a control oil mixture with the addition of menhaden oil, a rich source of LC n-3 PUFAs, or a control oil mixture, lacking LC n-3 PUFAs. Steers supplemented with menhaden oil observed improved insulin signaling pathways in the muscle, and increased activation of the mTOR-S6K1-4E-BP1 pathway, increasing whole body protein metabolism. Kamolrat et al. (44) utilized C2C12 cell culture line to identify the potential for increased protein synthesis *ex vivo.* Cultures supplemented with 50uM EPA had the highest detection of protein synthesis, and the least amount of protein breakdown measured by incorporation of l-[2,6-^{3}H]phenylalanine in disintegrations per minute (DPM) per nanogram of total trichloroacetic acid (TCA) precipitated protein or released into the medium in DPM per nanogram protein, respectively. The improvement of myocyte differentiation and decreased adipocyte differentiation coupled with increased protein synthesis by dietary LC

n-3 PUFA may supply an opportunity to attenuate the effects of wooden breast syndrome seen in commercial broiler lines.

CHAPTER I.

ENRICHING THE HEN AND CHICK DIET IN LONG-CHAIN OMEGA-3 POLYUNSATURATED FATTY ACIDS ALTERS CHARACTERISTICS ASSOCIATED WITH THE DEVELOPMENT OF WOODEN BREAST

Abstract

Consumption of long chain n-3 polyunsaturated fatty acids (LC n-3 PUFA) such as eicosapentaenoic acid (EPA; 20:5 n-3) and docosahexaenoic acid (DHA; 22:6 n-3) during development has been shown to decrease adipogenesis and promote muscle growth. We determined if the enrichment of EPA and DHA in embryo and post hatch would be effective in addressing the proposed causative changes found in the development of wooden breast. Cobb500 broiler-breeder hens consumed a diet enriched in either soybean (n-6 PUFA; 51% 18:2 n-6) or fish oil (3% fat wt:wt) for 28 days prior to fertilized eggs being collected. Resultant female chicks were fed starter diets enriched in either corn (CO; n-6) or fish oil (FO; n-3) (3.59%) within a 2 X 2 design (n=20/group) until 7, 9, 11, or 14 d of age. A subset of chicks from these hens were used to isolate primary cells for *in vitro* experiments and fed a commercial diet. Body weights and breast weights were recorded at each age. Pectoralis major and abdominal adipose tissue sections were taken to measure morphometrics, and gene expression (QPCR). Primary cells were cultured to measure triglyceride accumulation (Oil Red O), and gene expression (QPCR). Data were analyzed by ANOVA or t-test. Perinatal FO diet significantly increased average muscle fiber size, and the maternal FO diet reduced adipocyte size. The perinatal FO diet upregulated *FABP4*, the maternal FO diet increased *PAX7* abundance and the two diets interacted to increase

MYOD1 and *PDGFRβ* relative expression. Satellite cells from maternal FO accumulated significantly less triglyceride. These results suggest that enrichment of the maternal and perinatal diets in broilers may help to regulate the causative changes seen in wooden breast without compromising growth performance.

Introduction

Average per capita consumption in the US now approaches 99 lbs/year, which is almost four-fold higher than in 1950 (2). The use of genetic selection for rapid growth in broilers has allowed supply to meet demand, but has also resulted in negative consequences. Breast muscle myopathies (BMM) are the occurrence of spontaneous muscle abnormalities that are affiliated with the cellular dysfunction and metabolic imbalance triggered by the energy demand from the rapid rate of muscle growth. Breast muscle myopathies such as wooden breast (WB) and white striping (WS) are estimated to affect upwards of 90% of flocks of the most efficient broiler lines (12). These myopathies are very costly to the industry because they compromise the quality of the most valuable meat component of the bird. Affected muscle becomes fibrous and tough, and must be used for lower profit products (e.g., processed chicken nuggets) rather than sold as breast filets (45). These disorders are estimated to cost the industry almost $1 billion in economic losses per year. White striping is characterized by fibrotic striations that run in parallel to muscle fibers. Wooden breast is classified as the hardening of the *p. major* muscle, most commonly found on the proximal end of the muscle, and in severe cases, the hardening can be distributed throughout the filet.

Wooden breast syndrome's etiology has been challenging to identify in its entirety. However, the severity of these myopathies is most closely associated with growth rate inclusive of heavier body weights and thicker breast fillets (46, 47). A reduction in dietary energy and amino acid density to 90% of recommendations throughout the grow out period of broilers was able to reduce severity of myopathies studies, but did sacrifice growth efficiency (48). Additionally, wooden breast is not observed in slow growing broiler lines, further illustrating the relation of myopathy severity to growth rate. One of the most important contributors to the myopathy's development is the metabolic dysregulation that the broiler experiences because of the expedited growth rate. The fibers of *pectoralis major* undergo striking hypertrophic growth in a short period of time. This produces a greater amount of muscle, but also strains the ability for the bird to properly diffuse nutrients including oxygen between blood vessels and muscle fibers (49, 50). First, this has resulted in creating an oxygen deprived environment indicated by the increase in markers of hypoxia (51, 52) and production of reactive oxygen species (16). Secondly, it has been largely speculated that the rapid hypertrophic growth of *p.major* muscle results in fibers outgrowing the intramuscular connective tissue sacrificing the quality of the meat (53). The fibers and fiber bundles diameter grow beyond the endomysium and perimysium connective tissue support creating large intracellular spaces resulting in muscle damage (54). Muscle damage can negatively impact the chemical composition and processing, and inherently decrease the quality of the meat. The relationship between muscle morphology and development and growth of muscle is directly correlated to meat quality (55). Therefore, wooden breast associated hypertrophic growth coupled with fiber degeneration

and regeneration and subsequent metabolic dysregulation may be the biggest contributor to the impacts on meat quality.

Myofiber degeneration was first observed within the first two weeks of age in birds which further increased in severity as the birds aged (14). Developmentally, wooden breast has also been shown to be strongly associated with lipid metabolism dysregulation as early as two weeks of age. Genes associated with inflammation, fibrosis, and lipid metabolism are upregulated in affected vs. healthy breast muscle of market age birds of both disorders (16, 17). Macrophage infiltration and ectopic lipid accumulation in the extracellular matrix of myofibers from biopsies of young chicks (1-2 weeks of age) were found to be the earliest signs of subsequent myopathy at market age (14). Metabolomics data reflect similar lipid metabolism dysregulation. Breast of wooden breast-affected birds contained increased levels of lipid catabolism products including glycerol (18), as well as high levels of metabolites that reflect activity of vitamin B6, a vital coenzyme in lipid metabolism (56). Gene expression analysis also revealed increased expression of lipid metabolism genes at two weeks of age in chicks that eventually developed wooden breast (17). Included in this set of genes is *PPARγ*, which encodes a transcription factor that is described to be a master regulator of adipogenesis. Downstream targets of *PPARγ* include genes that promote fatty acid uptake, and several of these genes were also upregulated in wooden breast vs. healthy breast samples. These results suggest that the early pathology of the disease is associated with metabolic dysregulation, and that inappropriate adipogenesis within breast muscle may contribute to the pathology of these myopathies.

Satellite cells (SC) are responsible for developmental muscle growth and muscle regeneration in adult skeletal muscle. Also referred to as adult myoblasts, SCs are an undifferentiated stem cell population that contributes to hypertrophic muscle growth during development and to muscle regeneration in adults. They are named "satellite" cells because they reside in the perimeter of muscle fibers. Satellite cells contribute to hypertrophic growth by differentiating into myoblasts and fusing to form myofibers or by fusing with existing myofibers. In chicks, SCs proliferate during the late stages of embryogenesis and up until approximately two weeks after hatch, after which they become quiescent (27). Although the primary role of satellite cells is as precursors for myoblasts, they are multipotent stem cells that also have the ability to differentiate to adipocytes (57). Their plasticity is thought to be at its highest during late embryogenesis and just after hatch, when their rates of proliferation and differentiation are very high (28).

The skeletal muscle microenvironment in *p.major* also includes a population of undifferentiated stem cells known as fibroadipogenic precursors (FAPs) that are vital in muscle repair (58). While they remain quiescent when muscle is intact, they become active and activate myogenic progenitors in response to damage. More recently, they have been implicated in the production of adipocytes in the presence of ongoing muscle damage and subsequent degeneration. In addition, it has been suggested that in the presence of these adipocytes it may also reduce myogenesis (59). It should also be considered that FAPs may be a contributing source of adipocytes in *p.major* that contributes to the pathology of wooden breast and associated breast muscle myopathies.

We have shown previously that the dietary supplementation of marine oils in the maternal broiler-breeder hen diet effectively enriches the environment in embryo and the subsequent tissue fatty acid makeup of the chicks. A significant decrease in adiposity was observed in chicks that originated from hens fed a diet enriched with fish oil (37). We expanded on this understanding to test the hypothesis that enriching the embryonic environment and post hatch diet in LC n-3 PUFAs, EPA and DHA, would impact breast muscle growth and muscle morphology while also reducing inappropriate adipogenesis in *p.major*. We compared chicks originating from a hen fed fish oil to chicks originating from a hen fed soybean oil, a standard fat source used in commercial broiler-breeder diets that is composed n-6 PUFA. A subset of these chicks from both maternal origin were fed a standard commercial starter diet immediately after hatch to restrict any observed changes at the cellular level to maternal diet manipulation. A subset female broiler chicks from each maternal diet were evenly distributed among post hatch diets enriched in fish oil or corn oil to create a 2x2 feeding design. We found that supplementation of fish oil at the maternal and post hatch levels can impact skeletal muscle and adipocyte morphology and alter markers of muscle development and fatty acid metabolism. Additionally, at the cellular level LC n-3 PUFA in the maternal diet is able to significantly reduce triglyceride accumulation of satellite cells *in vitro.* Our results suggest that an increase in LC n-3 PUFA in the maternal and perinatal diets may be an effective way to address observed market age breast muscle myopathies during the developmental stages while maintaining growth.

Materials and Methods

Hen and chick husbandry and diets

Cobb 500 broiler chicks used in this study were produced at the University of Georgia poultry facility. Broiler-breeder hens were fed commercially-formulated soy- and fish-based diets with 3% fat (wt:wt) supplied by either soybean oil or fish oil. Soybean oil is a source of n-6 PUFA (57%, of which 51% is from linoleic acid (18:2 n-6) and is commonly used as a fat source in poultry diets. The fish oil that was used is extracted from menhaden and contains 18% EPA and 12% DHA. Hens were fed their assigned diet for a minimum of four weeks prior to collecting fertilized eggs. Fertilized eggs were incubated and hatched under standard commercial conditions, and feather-sexed at hatch. Within 24 hours after hatch, approximately 100 female chicks were transported to the University of Tennessee for a diet study in vivo. Chicks were fed one of two diets, using a 2 X 2 design: a starter diet supplemented with fish oil or corn oil (3.59%). Each oil was mixed into a starter diet containing a base fat content of 0.41% fat from corn oil; the diet was prepared and provided by the Poultry Feed Mill Facility at the University of Georgia. A separate set of fertilized eggs from the same set of hens was were incubated and hatched under standard commercial conditions at the University of Tennessee. These chicks, which were used to isolate primary cells for in vitro experiments, were fed DuMour commercial chick starter feed (Tractor Supply Co. Brentwood, TN) after hatch. Chicks were raised in standard housing conditions using a 23:1 light:dark cycle and fed *ad libitum*, until euthanasia.

Tissue collection

On 7, 9, 11, and 14 days of age, 20 birds (5/diet group) total were weighed and then euthanized by using the CO_2 method at UT in accordance with IACUC-approved protocol. The right pectoralis major (*p. major*) was dissected and weighed as an index of breast muscle growth. Total abdominal fat was harvested and weighed as a measure of adiposity. A longitudinal section of the left p. major was dissected from the center of the muscle, with half preserved for histology and half snap frozen for RNA isolation. For histology, approximately 1cm x 0.5cm x 0.5cm section of *p. major* was cut and placed on cardstock to preserve the orientation of muscle fibers. The breast sample was then placed in 10% neutral-buffered formalin for 24-48 hours, and then formalin was replaced with 70% ethanol for storage. Samples of the abdominal adipose depot was also be fixed overnight in 10% neutral-buffered formalin. Samples of *p. major*, abdominal adipose and liver to be used for RNA isolation were snap-frozen in liquid nitrogen and then stored at -80°C.

Breast muscle and adipose tissue histology

Histology, immunochemical staining and imaging software was used for morphometric analyses of muscle and adipose development. Tissues were embedded in paraffin, sectioned, and stained with hematoxylin and eosin (H&E) using standard protocols. One slide was made for each sample. Images of each slide were captured using the Advanced Microscopy Group (AMG) EVOS XL Core microscope (Fisher Scientific, Pittsburgh, PA). Three fields per chick were captured on each slide under 20X

magnification. Analyses was done using ImageJ (Version 1.8, National Institutes of Health) with MRI Adipocyte tools (https://github.com/MontpellierRessourcesImagerie/imagej_macros_and_scripts/wiki/Adipocytes-Tools). This tool kit identifies shapes (e.g., adipocytes or muscle fibers) in images and measures their areas. Fiber area was collected using a restriction that measurements must exceed 800um^2 while obtaining a minimum count of 600 fibers per slide. Fibers were then grouped into one of ten arbitrary bins separated by intervals of 250 and the frequency of fibers within each bin was determined. Similarly, adipocyte size was determined by collecting measurements of adipocytes that must exceed 350um^2 (60). The adipocytes were grouped into one of six arbitrary bins separated by intervals of 1500 and the frequency of adipocytes within each bin was determined. Subsequently, assuming circularity, adipocyte volume was calculated by finding the radius of each circle and then applying the following formula = ((4/3)*(Π)*(adipocyte radius^3)). In order to determine cell number in the visceral fat pad collected, the units from the volume of each adipocyte was converted to mL. The mass of each individual adipocyte was determined by assuming the density was 0.9g/mL and the final adipocyte mean weight for each sample was calculated. The total fat pad mass collected at termination was divided by the mean adipocyte weight per each sample to approximate total number of adipocytes within the mass.

Real time PCR assay

Total RNA was isolated from breast muscle (~20-25 mg) and from primary satellite cells using TRIzol (Invitrogen Carlbad, CA) and purified using Zymo Direct-zol Miniprep kit (Zymo Research Irvine, CA), following the manufacturers' protocols. RNA was

quantitated via spectrophotometry and quality was assessed using gel electrophoresis. The iScript cDNA synthesis kit (Bio-Rad Hercules, CA) was used to synthesize cDNA from ~500ng of total RNA. Successful cDNA synthesis was confirmed by PCR amplification of a housekeeping gene prior to performing QPCR. Quantitative polymerase chain reaction was performed using BioRad SYBR Green Universal Master Mix (Hercules, CA) and the CFX96 Touch Real-Time PCR Detection System (BioRad). The cDNAs were pooled and used to make 5 serial dilutions to produce a standard curve to verify the efficiency of each primer pair. RNase free water was used as a negative control, and each sample was run in duplicate. Expression levels of genes of interest were normalized to the expression of the housekeeper gene. Genes of interest include peroxisome proliferator activated receptor gamma (*PPARγ)*, lipoprotein lipase (*LPL*), fatty acid binding protein 4 (*FABP4*), paired box 7 (*PAX7),* myogenic differentiation 1 (*MYOD1*), platelet-derived growth factor receptor alpha and beta (*PDGFRα/β).* *TBC* was used as a reference gene for primary cells, which *HPRT1* was used as a reference gene for breast muscle samples.

Tissue collection and satellite cell isolation four day-old chicks

3 chicks from each maternal diet treatment at 4 days of age were euthanized for isolation of primary satellite cells. Breast muscle (p, major) from each chick was dissected under sterile conditions and pooled (breast/chick, 3 chicks/diet). Both right and left breast filets were removed carefully, leaving behind gross connective tissue and ossified rib or keel bone. Filets were placed in DMEM/F12 1% P/S media without serum until completion of dissection. Filets were placed in 1X PBS and cleaned of visible debris, connective tissue, and feathers. After cleaning, the filets were minced into small pieces and cleaned further

of any remaining tissue debris. The minced pieces were then transferred to a 50mL conical tube containing 10mL of 0.05% Trypsin-EDTA (MilliporeSigma St. Louis, MO). An up and down motion to mix and continued mincing with large scissors was used to breakdown the pieces in the 50mL conical tube. The solution containing the minced pieces was incubated at 37°C for 20 minutes. The pieces were collected via low speed centrifugation and removed of the supernatant and resuspended in 1mL of growth media (GM), 10% Fetal Bovine Serum in Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F12; ThermoFisher Waltham, MA). The tissue was pipetted vigorously to release the cells from the digested tissue. The tissue and solution, containing the satellite cells, was filtered through a 70um filter in two disbursements. The cells were pelleted by low speed centrifugation and resuspended in red cell lysis buffer (MilliporeSigma St. Louis, MO) and set to incubate at room temperature for 5 minutes. The reaction was quenched and the cells were resuspended in 5mL of growth media. The cell pellet was re-formed via low speed centrifugation and resuspended in 1mL of growth media. Cells were counted using a hemocytometer and Corning Cell Counter (CystoSMART, The Netherlands) after staining with Trypan blue (1:1).

Satellite cell purification and growth period

The cells were plated at 2.2 x 10^6 cells per 100mm plastic tissue culture plates, and placed in a standard humidified cell culture incubator (37°C, 5% CO_2) for two hours. Satellite cells do not adhere to plastic, and will remain in the supernatant of the growth media. After the conclusion of the incubation period, the supernatant from each plate was transferred to 100mm tissue culture plates with gelatin (2%; (MilliporeSigma St. Louis,

MO) coated wells. The cells that did adhere to the 100mm plastic tissue culture plates were grown to 80% confluence in GM, labeled “plastic adherent satellite cells” (PASC) and used for downstream applications.

Satellite cells which were also labeled “non-adherent satellite cells” (NASC) referring to their inability to attach to plastic culture plates were grown to 80% confluence in myogenic growth media (MGM), 10% Chicken Serum in Dulbecco’s Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F12; ThermoFisher Waltham, MA). After reaching confluence, the 100-mm culture dish was washed with 1X PBS, and 5mL of 0.25% Trypsin-EDTA (MilliporeSigma St. Louis, MO) was used for each 100-mm culture dish. Each plate was placed in a standard humidified cell culture incubator (37°C, 5% CO_2) until cells were released from the plate (~5 minutes). The Trypsin-EDTA and cell mixture was used to repeatedly pipette forcefully in an effort to disrupt cell colonies requiring mechanical disruption. A total of 10mL of growth media was added to each plate to quench the reaction of Trypsin-EDTA, and the supernatant was used to continually wash the plate of any remaining cells and transferred to a 15mL conical tube. The supernatant was spun at centrifugation speed of 1000rpm for 10 minutes to collect the cells in a pellet. The remaining supernatant was aspirated, and the cell pellet was resuspended in 1mL growth media. Cells were counted using a hemocytometer and Corning Cell Counter (CystoSMART, The Netherlands) after staining with Trypan blue (1:1). Cells were plated and grown in MGM to ~60-70% confluence and cells were plated and grown in adipogenic induction media.

Genomic DNA isolation and assessment of relative mitochondrial density

Genomic DNA was isolated from satellite cells at 80-90% confluence and from 14 day *p. major* tissue for relative assessment of mitochondrial density. Tissues were homogenized and pelleted cells were resuspended in lysis buffer [1M TRIS, 0.5M EDTA, 20%SDS, 1M NaCl] containing 5µL of proteinase K per mL of lysis buffer. The cells were lysed in a 2mL tube by passing the cell mixture through a BD syringe and 26G needle (Medsitis Hattiesburg, MS) 15 times. The lysates were placed in a water bath warmed to 56°C and shaken at 151 rpm for 2 hours. The samples were then spun at 14,000g for 20 minutes at 4°C. The clear supernatant was saved and transferred to clean 1.5mL tube. An equal volume of isopropanol was added to the clear supernatant and then spun at 14,000g for 30 minutes at 4°C. The supernatant was discarded and the DNA pellet was washed in 1mL 70% Ethanol. The pellet was spun at 14,000g for 10 minutes at 4°C. This steps was repeated to remove all salts. The ethanol was then discarded and the pellet was left to dry for 5-10 minutes. 50uL of DNAse/RNAse free water was heated to 55°C and added to each sample to resuspend the cell pellet. Total gDNA was quantitated via spectrophotometry verifying optical density at 260nm using an Amersham Ultra Spec 1300 Pro Spectrophotometer (Amersham Buckinghamshire, England). Quality of gDNA was confirmed using gel electrophoresis. Mitochondrial density was assessed indirectly based on the abundance of a mitochondrial gene (cytochrome C oxidase (*COX1*)) relative to a nuclear gene (*18S*). Abundance of each gene in gDNA was determined using QPCR. Ct values were converted from exponential to linear values using $2^{-\Delta Ct}$ and then the ratio of *COX1*/*18S* was calculated and averaged across cells from the two diet groups or averaged across tissues from the four diet combinations.

Evaluating adipogenic potential of satellite cells

NASCs were grown to 80% confluence with MGM on 2% gelatin coated 100mm tissue culture plates and then trypsinized, and counted. Cells were plated with adipogenic induction media onto 2% gelatin coated 12-well tissue culture plates. Adipogenesis was induced by substituting growth media with DMEM containing 10% chicken serum and supplemented with albumin-bound oleic and linoleic acids (60 uM total final concentration; MilliporeSigma St. Louis, MO). After 120 hours, adipogenesis was quantified by measuring triglyceride accumulation. Oil red O was used to stain triglycerides. Briefly, the wells were washed with 1X PBS, and fixed with 10% buffered Formalin. The wells were washed with dH_2O and Isopropanol (60%) was added for a 5 minute incubation period and removed. The Oil red O stain (working solution 60:40 stain:dH_2O) was added once the wells were completely dried, and set to incubate for 20 minutes at room temperature. The stain was removed and each well was washed with dH_2O 4 times. Images of each well were captured using the Advanced Microscopy Group (AMG) EVOS XL Core microscope (Fisher Scientific, Pittsburgh, PA). All of the water was taken off and replaced with Isopropanol (100%). After wells incubated for 5 minutes at room temperature, 200uL was extracted from each well and transferred to a 96-well plate. Quantification of adipogenesis was performed spectrophotometrically using BioTek Epoch 2 Microplate Spectrophotometer (ThermoFisher Waltham, MA) via optimum absorbance at 510nm. Isopropanol (100%) was used as a blank.

Statistical Analysis

The experimental design was a completely randomized design with factorial treatments with chick as the experimental unit. Statistical analyses were performed in SAS 9.4 (SAS Institute Inc., Cary, NC, USA). Prior to analyses, independence of observations, normality of individuals, and equal variance were verified using PROC UNIVARIATE. When needed, log transformations were used for data that was found to be non-normal. Data collected were analyzed using a mixed model ANOVA within PROC GLIMMIX with terms for maternal diet, starter diet and their interaction using a p-value of < 0.05 as criterion for significance. Data collected from cells from Oil Red O staining, and QPCR were compared using two-sample t-test assuming unequal variances ($P < 0.05$).

Results

Performance and body composition

Instituting a method to address the severity or incidence of WB must also maintain the current standard of growth in modern boilers. The impacts of the maternal and perinatal diets on body composition were explored by morphometric analysis of *p. major* and abdominal adipose as both of these areas have problems associated with the negative implications of rapid growth. Stained images of muscle fibers cut in cross section were evaluated for changes in hypertrophic growth. The perinatal diet enriched in corn oil favored smaller fibers within the first two bins. Chicks consuming the diet enriched in fish oil were shown to have a greater number of larger fibers within bins 5, 6, and 7 (Figure 1.1). The maternal and perinatal diets did not interact to affect adipocyte volume (Figure 1.2A) or adipocyte number (Figure 1.2B). The maternal fish oil diet influenced adipocyte

morphology significantly, favoring a greater percentage of smaller adipocytes coupled with significantly fewer, larger adipocytes found in bins 5 and 6 (Figure 1.3).

Gene expression analysis and mitochondrial density

Identifying the potential mechanisms for the difference in muscle fiber morphology was done by evaluating relative expression of markers associated with myogenic commitment and differentiation. The maternal fish oil diet chicks had a higher expression of the early myogenic marker, *PAX7* (Figure 1.9). As illustrated in Figure 1.10, the maternal and perinatal diets interacted to effect *MYOD1* expression. In chicks that originated from soybean oil hens and were fed a corn oil enriched diet, *MYOD1* expression was lower when compared to all other diets ($P < 0.05$). Expression of *PPARγ* (Figure 1.4) and *LPL* (Figure 1.5) did not differ between diet groups. However, *FABP4* expression was observed to be higher due to the fish oil enriched perinatal diet (Figure 1.6; $P < 0.05$). *PDGFRα* is an important marker of fibroadipogenic progenitor cells, and did not differ in expression amongst the four diet groups (Figure 1.7; $P = 0.9877$). *PDGFRβ*, a marker of pericytes that assist with myogenesis and angiogenesis, expression increased due to an interaction between the maternal and starter diets (Figure 1.8; $P = 0.0368$). The ratio of mitochondrial DNA to nuclear DNA did not differ among diet groups (Figure 1.11).

PASC triglyceride accumulation and mitochondrial density

The formation and accumulation of lipid droplets is dependent of adipocyte metabolism and indicative of adipogenic activity. Lipid droplets were stained with Oil Red O after SCs were grown in adipogenic induction media for 5 days to quantify total triglyceride accumulation. The fatty acid composition of the maternal diet impacted

triglyceride accumulation (Figure 1.12A). The PASCs by the maternal fish diet accrued less triglyceride than PASCs influenced by the maternal soybean diet ($P \leq 0.05$). The maternal fish oil PASCs observed a greater ratio of mitochondrial DNA to nuclear DNA, but did not differ from the maternal soybean oil PASCs (Figure 1.14; $P = 0.20$).

NASC triglyceride accumulation and adipogenesis marker expression

Similarly seen in the PASC population, NASCs accumulated less triglyceride in the maternal fish oil when compared to maternal soybean diet group (Figure 1.12B; $P < 0.05$) As shown in Figure 1.13, the differences in expression of *PPARγ* between the two diets were numerically different, but did not meet the criterion for statistical significance ($P = 0.20$).

Discussion

Modern day broiler efficiency is essential for continuous production with minimal cost. However, broiler growth efficiency has also introduced unwanted side-effects in the form of BMM compromising the most profitable portion of the bird. The incidence and severity of one of the most common myopathies, WB, is most closely associated with the level of broiler efficiency. The underlying cause of the myopathy has yet to be identified, but it is thought to be tied to the rapid hypertrophic growth of muscle fibers that outgrow their supply lines for gas exchange and metabolism. Additionally, the normal physiological processes of muscle repair appear to be disrupted. Rather, the damaged muscle leads to muscle degeneration and scarring coupled with infiltration of fat and connective tissue. Within the first two weeks of age, broilers found to have WB at market age had the earliest signs of myopathy development in the form of muscle degeneration and ectopic

adipogenesis in fibers. There is ordinary only a small population of mature adipocytes in highly glycolytic and lean muscles such as pectoralis major, suggesting there may be alternative sources at work for ectopic lipid accumulation to occur. The cell population in *p. major* also includes fibroadipogenic precursors (FAPs) and SCs. Both play essential roles in muscle regeneration after damage, but could also be contributing sources of mature adipocytes. Limiting lipid accumulation by minimizing inappropriate adipogenesis in *p.major* of broilers in early stages of development may be an effective way to lessen severity or incidence of WB. Previous studies performed during highly influential periods of development in chicken, namely during embryonic development and immediately post hatch, have shown the ability to decrease adiposity in chicks (34, 37). Enrichment of the embryonic environment and post hatch diet in LC n-3 PUFA was effective in manipulating adipogenesis in fat depots. This study investigated the effects of supplementing the maternal diet as well as the perinatal diet with fish oil vs soybean or corn oil on adipogenesis and myogenesis *in vivo* and *in vitro* while ultimately conserving broiler growth rate.

Incorporating fish oil in the perinatal diet impacted muscle fiber morphology in *p.major* and favored larger fibers when compared to corn oil diet chicks. However, increased muscle growth in early stages of development may be of benefit to the broiler. While it may be suggested that a greater frequency of smaller fibers would be more favorable in early stages of development, the same could also be said for adiposity. A study conducted by Tompkins et al. (61) fed broiler-breeders a diet enriched with 2.3% fish oil or soybean oil for four weeks and assessed the changes in body composition of chicks from

each diet group. The maternal fish oil diet significantly improved both body weight and body weight gain of chicks at the finisher stage. Early assessment of body fat showed that the fish oil group had a high percentage, but had a higher percentage of lean mass was detected in this same group at day 42. Similar trends may also be observed in skeletal muscle in chicks fed or derived from maternal fish oil diets at market age. The maternal fish oil diet also impacted white adipose tissue morphology by favoring smaller adipocytes when compared to chicks from the maternal soybean oil diet. Differences in lipid droplet size have been shown to influence lipid metabolism and are markedly impacted by changes in expression of localized proteins associated with storage and lipolysis. A study done by Sawada et al. (62) found that overexpression of Perilipin A, a lipid droplet associated protein, reduces the expression of lipid droplet protein FSP27 effectively decreasing lipid droplet size and promoting a brown adipose tissue-like phenotype. Further insight into the mechanisms by which fish oil is able to promote smaller adipocyte size including the associated proteins in localized areas that may have alternative expression due to diet.

The expression of *PPARγ*, *LPL*, and *FABP4* are markers of adipogenesis and fatty acid metabolism that were observed in other studies to have heightened expression in as early as two weeks of age in chicks who later developed WB when compared to healthy broilers (17). The difference in relative mRNA abundance of *FABP4* was significant between maternal fish and soybean oil diet groups. Historically, *FABP4* has been used as a marker of adipogenesis. More recently, it has also been identified in skeletal muscle in human with an increase in expression after a period of endurance training. Previous observations have confirmed the ability for enhanced fatty acid oxidation in endurance-

trained individuals (63). In addition, fatty acid binding proteins have been shown to play essential roles in transport of fatty acids to the mitochondria for oxidation, including *FABP4.* The changes in expression may also speak to the metabolic adaptation occurring within the skeletal muscle during endurance training. Similarly, the differences in expression of *FABP4* based on diet may reflect the changes in metabolism occurring in muscle related to increased hypertrophic growth or a heightened ability for fatty acid oxidation coupled with increased mitochondrial biogenesis. The relative expression of *PAX7* was significantly different as the maternal fish oil group observed a greater relative expression. The expression of *PAX7* is essential for the commitment of myogenic precursors, namely satellite cells, to commit to the next steps of myogenesis while also maintaining a quiescent SC pool to assist in hypertrophic growth or repair. The expression of *MYOD1* is an early myogenic differentiation marker and is also essential in the formation of myotubes and mature myofibers. Halevy et al. (64) confirmed this occurrence in chicks immediately post hatch where the expression of *PAX7* is very high within the first day of hatch and begins to taper off as the expression of *MYOD1*, the marker of differentiation, begins to increase. They concluded that *PAX7* expression is able to identify SCs and their progeny that can enter the cell cycle or remain quiescent. The maternal and starter diets interacted to increase the expression of pericyte marker, *PDGFRβ*. Pericytes have been classified as mesangioblasts that contribute to hypertrophic muscle growth postnatally, and support vascularization (65, 66). The increase in myogenic markers, *PAX7* and *MYOD1*, and pericyte marker, *PDGFRβ*, may help to further explain the increase in

hypertrophic growth, and potentially an increased blood supply seen in chicks supplemented with fish oil in the starter diets.

The skeletal muscle microenvironment includes an additional population known as FAPs which we identified as PASCs. They have been identified to have the ability to differentiate into mature adipocytes, potentially stunting myogenesis and contributing to ectopic lipid accumulation. The maternal fish oil diet appeared to potentially reduce FAPs propensity to terminally differentiate into adipocytes. Recent reports have suggested that an increase in mitochondrial density may be associated with an increased capacity for fatty acid oxidation and overall energy metabolism (67). The difference between maternal diet cell groups' mtDNA/nDNA ratio was not statistically different, but the maternal fish oil group was greater when compared to the maternal soybean oil cells. Relatively little is known about regulation of SCs adipogenesis *in vivo*. Conversely, *in vitro* studies have confirmed the ability for SCs to undergo alternative terminal cell differentiation. Shefer et al. (68) demonstrated SCs plasticity by harvesting mouse extensor digitorum longus muscle and culturing single myofibers in serum-rich growth media. Satellite cells cultured from single myofibers exhibited mesenchymal stem cell-like characteristics because clones of these SCs were shown to differentiate into either mature myocytes or adipocytes under conditions suitable for both muscle and fat development. Primary chicken SCs treated *in vitro* have been shown to exhibit similar changes in cell lineage commitment. In chicks, a study by Powell et al. (69) revealed that access to feed and its interaction with delayed hatch times influenced SC commitment to favor adipogenesis, increasing intramuscular adipose tissue deposition. Previous studies have highlighted that immediate post-hatch

20% feed restriction over the duration of two weeks results in an increased intramuscular adipose tissue deposition (70). In addition, Velleman et al. (71) found an increase in genes that serve as regulatory factors for adipogenesis such as *PPARγ* and *C/EBPα* in chicks who underwent feed restriction within the first week post-hatch. Velleman et al. (72) later revealed that there was also an association with increased expression of *PPARγ,* in addition to *C/EBPα* and stearoyl-CoA desaturase both *in vivo* of isolated SCs and *in vitro* from *p. major* breast tissue from a growth selected turkey line when compared to a random-bred control line. The SCs we identified as NASCs showed to have a significantly lower triglyceride abundance in the maternal fish oil cells. However, our gene expression analysis reveals this may not be due to the expected lowered expression of adipogenic master regulator, *PPARγ*.

In summary, our data demonstrate that the consumption of fish oil provided by the maternal diet and the perinatal diet may be a strategy with which to address extensive and harmful hypertrophic growth and inappropriate adipogenesis associated with WB and affiliated BMM. We were able to effectively change the muscle fiber morphology while also limiting triglyceride accumulation in breast muscle cells that may play a vital role in ectopic lipid accumulation in myopathies. This study was limited to the first two weeks of life, and further studies should be done to determine if fish oil supplementation can alleviate the incidence and/or severity of WB at market age. This study also emphasizes the importance of the maternal and perinatal diets and their impacts on long-term physiology of the offspring.

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Appendix

Table 1.1 Composition of Fish Oil (FO) and Soybean Oil (SO) broiler breeder diets fed to hens for 28 d

Item	FO	SO
Ingredients, %		
Corn	63.16	63.16
Soybean meal (48%)	11.85	11.85
Wheat midds	8.00	8.00
Limestone	7.90	7.90
Pro-pak	5.00	5.00
Fish oil	2.30	-
Soybean oil	-	2.30
Mono calcium phosphate	0.60	0.60
Termin-8	0.30	0.30
Salt	0.20	0.20
Vit Pre-mix	0.20	0.20
Bicarbonate of Soda	0.15	0.15
DL-Methionine	0.14	0.14
Choline	0.12	0.12
TM Pre-mix	0.08	0.08
Nutrients		
ME, kcal/kg	2938	2938
CP, %	15.20	15.20
Calcium, %	3.5	3.5
Total phosphorus	0.61	0.61
Available phosphorus	0.43	0.43

Table 1.2 Composition and calculated nutrient content of Fish Oil (FO) and Corn Oil (CO) basal diet fed chicks 0-14d

Item	FO	CO
Ingredients, %		
Corn, Grain	60.34	60.34
Soybean meal (48%)	31.99	31.99
Dical. Phos	1.73	1.73
Corn oil	0.39	3.84
Fish oil	3.45	-
Limestone	0.5	0.5
Common salt	0.22	0.22
DL-Methionine	0.27	0.27
Vitamin premix	0.24	0.24
L-lysine-HCL	0.16	0.16
Threonine	0.05	0.05
Mineral premix	0.08	0.08
Coban-90	0.05	0.05
Nutrients		
ME, kcal/kg	3008	3008
Crude protein %	21.00	21.00
Dig-Lysine %	1.18	1.18
Dig-Methionine %	0.59	0.59
Dig-TSAA %	0.88	0.88
Dig-Threonine %	0.77	0.77
Ca%	0.90	0.90
avP%	0.45	0.45

Table 1.3 Effect of dietary enrichment on growth performance of broiler chicks from hens fed SO or FO for 28d and starter diets containing either CO or FO for 7, 9, 11, and 14d

Maternal Diet	SO		FO			Effect		
Starter Diet	CO	FO	CO	FO	SEM	Maternal	Starter	Maternal x Starter
							P-value	
Weight gain (g) 7d	108	104.83	104.26	85.35	6.1638	0.0780	0.0923	0.2198
Weight gain (g) 9d	149.42	157.53	145.97	134.56	9.5389	0.1851	0.8646	0.3215
Weight gain (g) 11d	237.87	255.67	229.62	193.37	11.3431	0.0067	0.4281	0.0299
Weight gain (g) 14d	329.65	358.24	346.88	344.41	15.9513	0.9165	0.4250	0.3448
Breast weight (g) 7d	12.5912	11.5852	9.6397	8.5572	1.4400	0.0542	0.4794	0.9799
Breast weight (g) 9d	19.9932	20.8732	18.0752	16.1652	1.8617	0.0941	0.7856	0.4645
Breast weight (g) 11d	32.4560	34.5136	18.0752	16.1652	2.1786	0.0025	0.2194	0.041
Breast weight (g) 14d	52.3084	51.7052	50.8788	53.3448	5.8146	0.9858	0.8747	0.7952

Table 1.3 continued

Maternal Diet	SO		FO			Effect		
Starter Diet	CO	FO	CO	FO	SEM	Maternal	Starter	Maternal x Starter
							P-value	
Fat pad weight (g) 7d	0.8734	0.831	0.8512	0.719	0.1324	0.6192	0.519	0.7389
Fat pad weight (g) 9d	1.5166	1.7888	1.8048	1.0486	0.1971	0.2683	0.2372	0.019
Fat pad weight (g) 11d	2.4772	3.3784	2.412	1.9724	0.3385	0.0451	0.505	0.651
Fat pad weight (g) 14d	3.6474	4.334	4.4862	3.77	0.4848	0.7805	0.9761	0.1673
Relative breast weight (%) 7d	0.0699	0.0698	0.063	0.0636	0.0072	0.4143	0.9762	0.9556
Relative breast weight (%) 9d	0.0842	0.0955	0.0909	0.0973	0.0054	0.4665	0.1105	0.583
Relative breast weight (%) 11d	0.1104	0.1111	0.1037	0.0894	0.0063	0.043	0.3004	0.2551
Relative breast weight (%) 14d	0.1369	0.1271	0.1251	0.1347	0.012	0.8618	0.9956	0.4351

Table 1.3 continued

Maternal Diet	SO		FO			Effect		
Starter Diet	CO	FO	CO	FO	SEM	Maternal	Starter	Maternal x Starter
							P-value	
Relative fat pad weight (%) 7d	0.00498	0.00507	0.00539	0.00532	0.0008	0.7023	0.988	0.9119
Relative fat pad weight (%) 9d	0.00669	0.00837	0.00894	0.0059	0.001	0.916	0.4786	0.0094
Relative fat pad weight (%) 11d	0.00821	0.01053	0.00803	0.00877	0.0009	0.3112	0.1151	0.4031
Relative fat pad weight (%) 14d	0.0095	0.01061	0.01119	0.00949	0.001	0.7971	0.7897	0.2071

Table 1.4 Genes and primers used for qPCR

Gene	Forward Primer	Reverse Primer
PPARγ	GTCTTGCAGTTGGAATGTCAC	ATGTCGCTGGAAATCTCTGC
LPL	TACAGTCTGGGTGCTCATGC	CCACAAAGTCAGCATCATCC
FABP4	ACCATCAGATCAGAAAGTACC	GCCATTGTCTAGGGTTATGA
PDGFRα	GCTTCCTGATGTGGAATGGT	TGGCTTTCCACCATATTCCT
PAX7	Commercial Source[1]	Commercial Source[1]
MYOD1	AGCAGCTACTACACGGAATC	GGACTCCCTTCAGCTACAG
PDGFRβ	GCCAGGAACGTCCTCATCTG	TCTCTGGGGCCATCCACTTA
COX1	AAACACCGTAGATGCCCAAG	TAGGGGGAGGTCTGTTGTTG
18S	CCATGGTGACCACGGGTAAC	GGATGTGGTAGCCGTTTCTCA
TBC	TGGCTGACACTGCTCTTGTC	CTCCTTCCAGCAACTGATCC
HPRT1	Commercial Source[1]	Commercial Source[1]

[1]QuantiTect (Qiagen; Germantown, MD; primer sequences are proprietary)

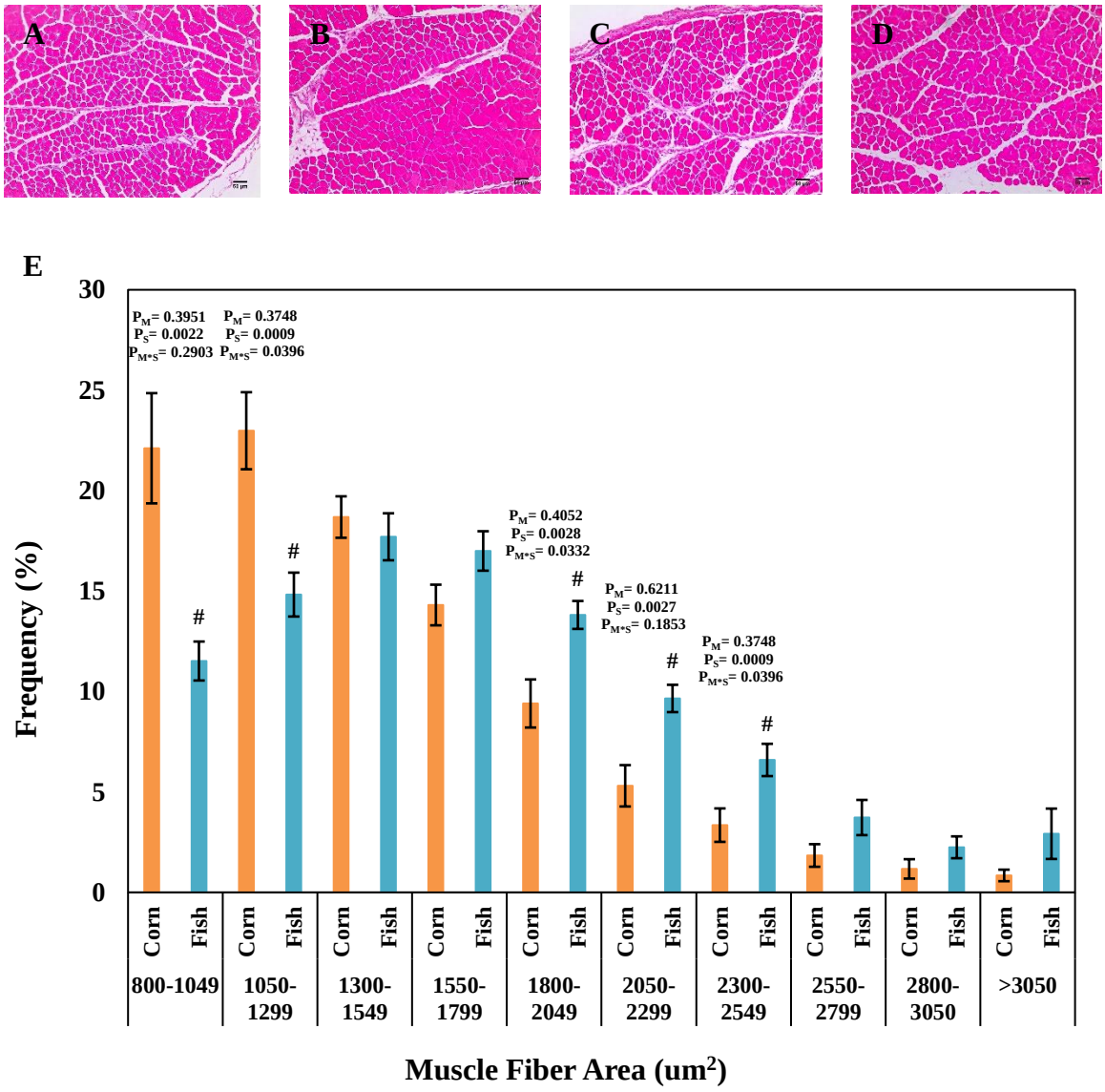
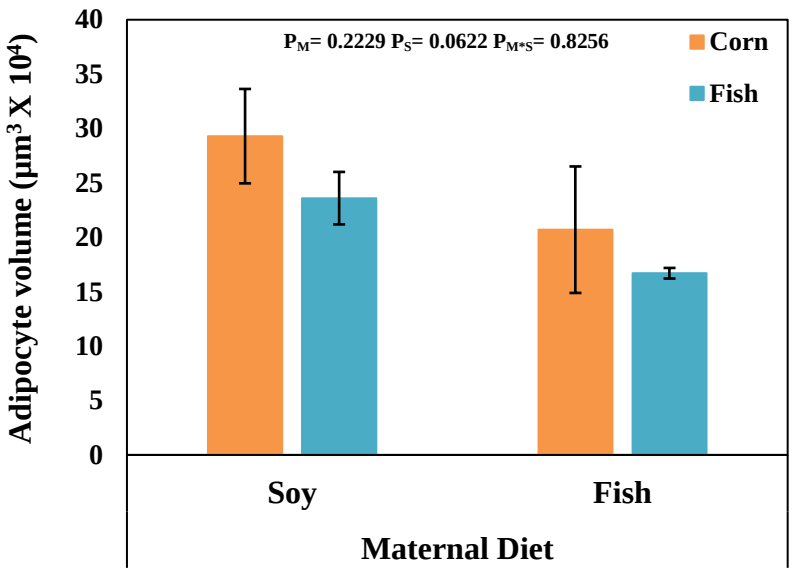


Figure 1.1 Representative H&E-stained images of *p. major* from SO hen chick fed CO (A), SO hen chick fed FO (B), FO hen chick fed CO (C), and FO hen chick fed FO (D). Muscle fiber areas (E) were measured from H&E-stained images using ImageJ (Version 1.8, National Institutes of Health). On average, 600 fibers were measured per chick. Areas were grouped into arbitrary bin sizes and frequencies counted within each bin. Frequencies within each bin were compared using two-way ANOVA with terms for maternal diet (PM), starter diet (PS) and interaction between the two diets (PM*S). Values represent average frequency of cells within each bin and compare the effect of FO and CO starter diets, + SEM; # = $P < 0.05$; n=5/diet group

A



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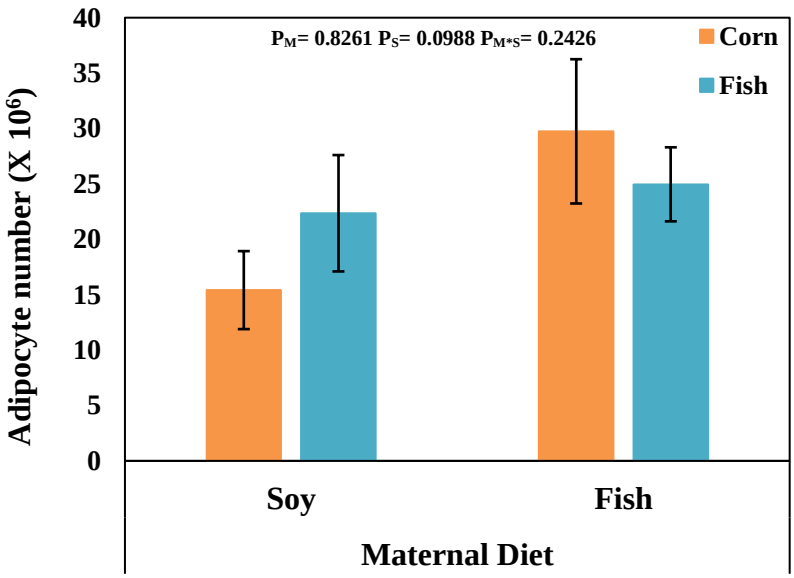

Figure 1.2 Adipocyte volume and number were compared using two-way ANOVA with terms for maternal diet (P_M), starter diet (P_S) and interaction between the two diets (P_{M*S}) ($P \leq 0.05$). (A), average adipocyte volume (µm^3 X 10), $\pm$ SEM; (B) average adipocyte number (X 10^6), $\pm$ SEM; FO vs. SO vs. FO vs. CO; n=5/diet group

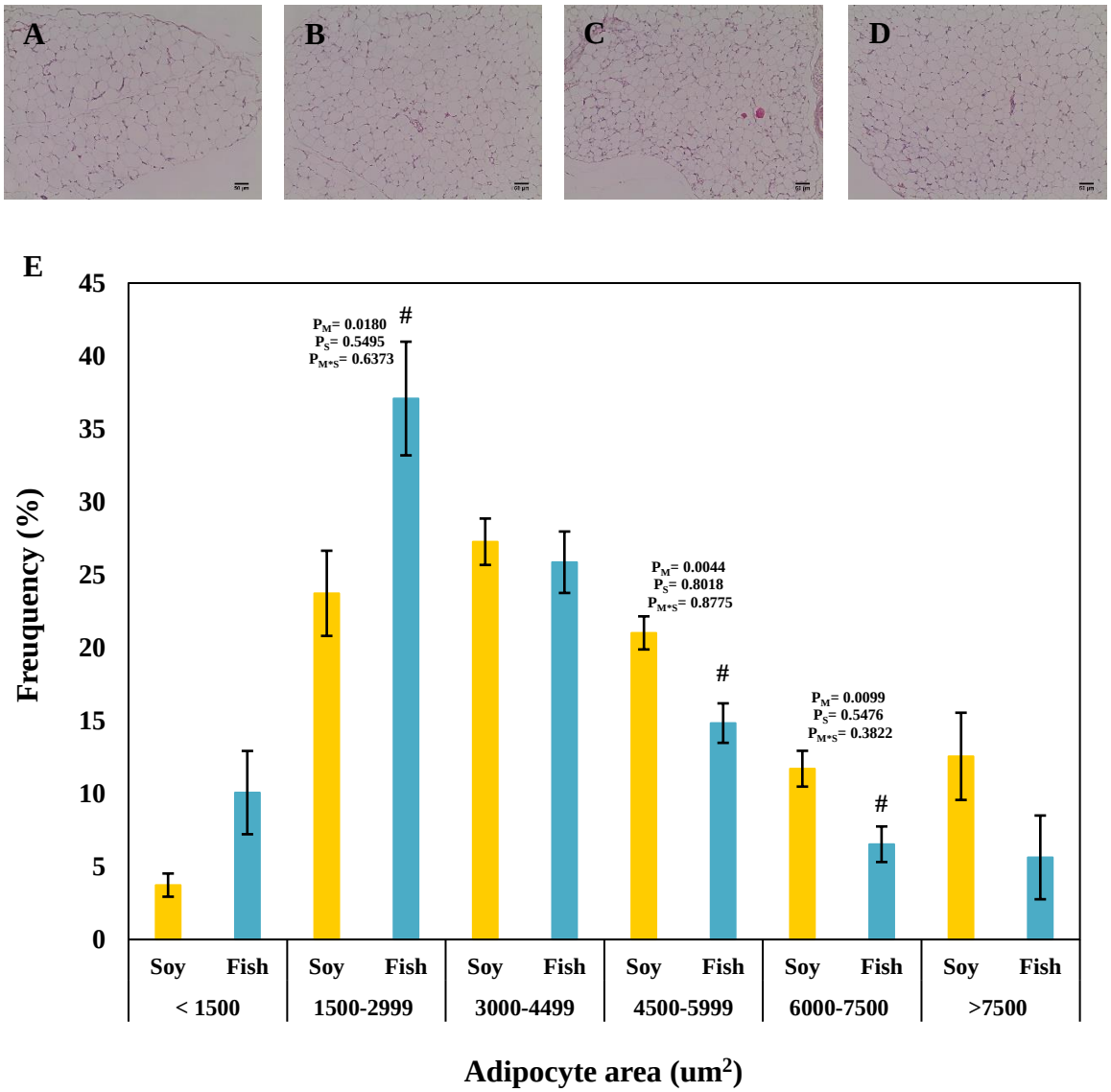

Figure 1.3 Representative H&E-stained images of abdominal adipose tissue from SO hen chick fed CO (A), SO hen chick fed FO (B), FO hen chick fed CO (C), and FO hen chick fed FO (D). Adipocyte areas (E) were measured from H&E-stained images using ImageJ (Version 1.8, National Institutes of Health). On average, 600 adipocytes were measured per chick. Areas were grouped into arbitrary bin sizes and frequencies counted within each bin. Frequencies within each bin were compared using two-way ANOVA with terms for maternal diet (P_M), starter diet (P_S) and interaction between the two diets (P_{M*S}). Values represent average frequency of cells within each bin and compare the effect of FO and SO maternal diets, $\pm$ SEM; # = $P \leq 0.05$; n=5/diet group

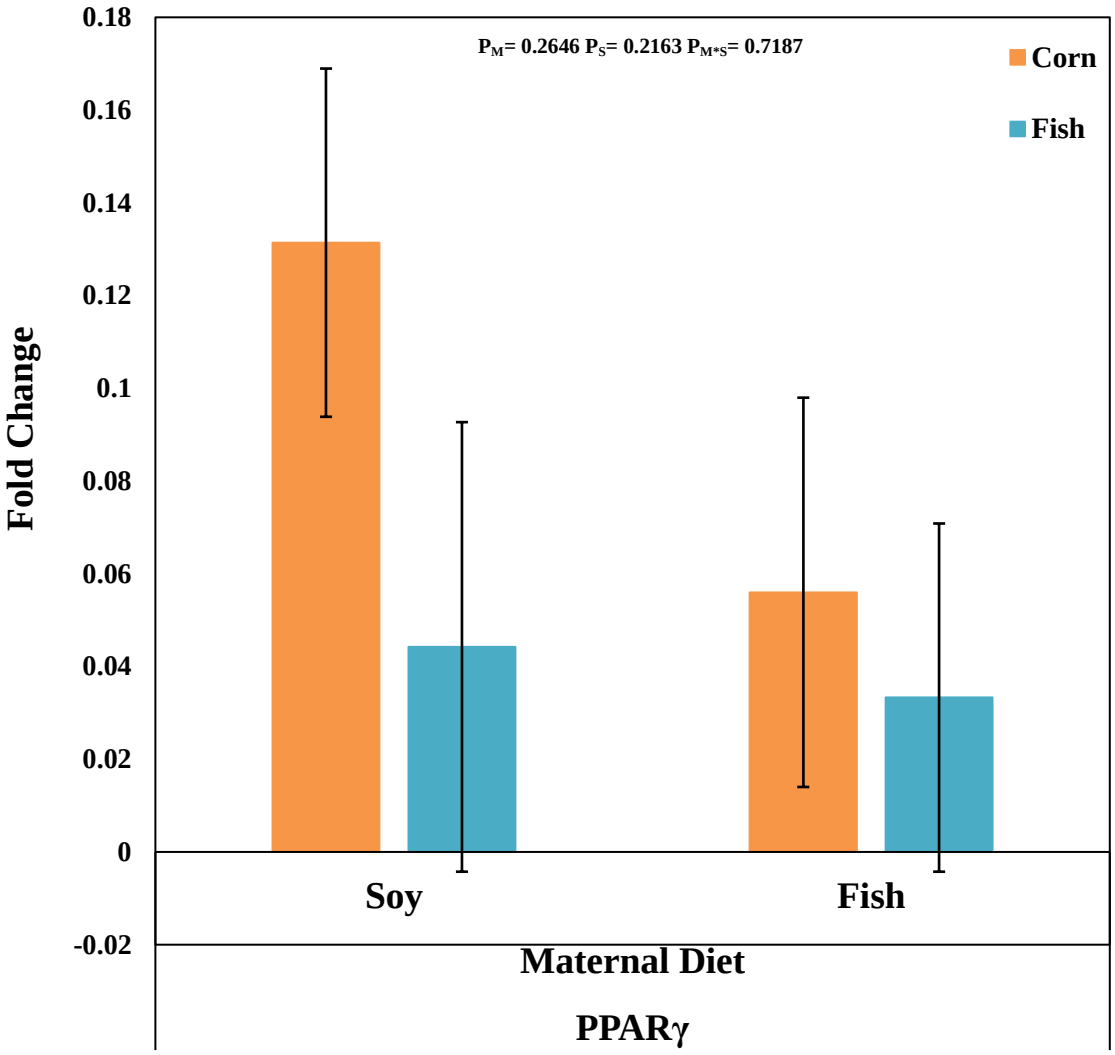

Figure 1.4 Expression of gene involved in adipogenesis in *p. major* of chicks from SO and FO hens fed FO or CO at 14d of age. *PPARγ*, Peroxisome proliferator-activated receptor gamma; Expression levels were normalized to expression of a reference gene (*HPRT1*) and converted to linear values using $2^{-\Delta Ct}$. Values were compared using two-way ANOVA with terms for maternal diet (P_M), starter diet (P_S) and interaction between the two diets (P_{M*S}) ($P \leq 0.05$). Values represent average relative mRNA abundance, $\pm$ SEM; FO vs. SO vs. FO vs. CO; n=3-5/diet group.

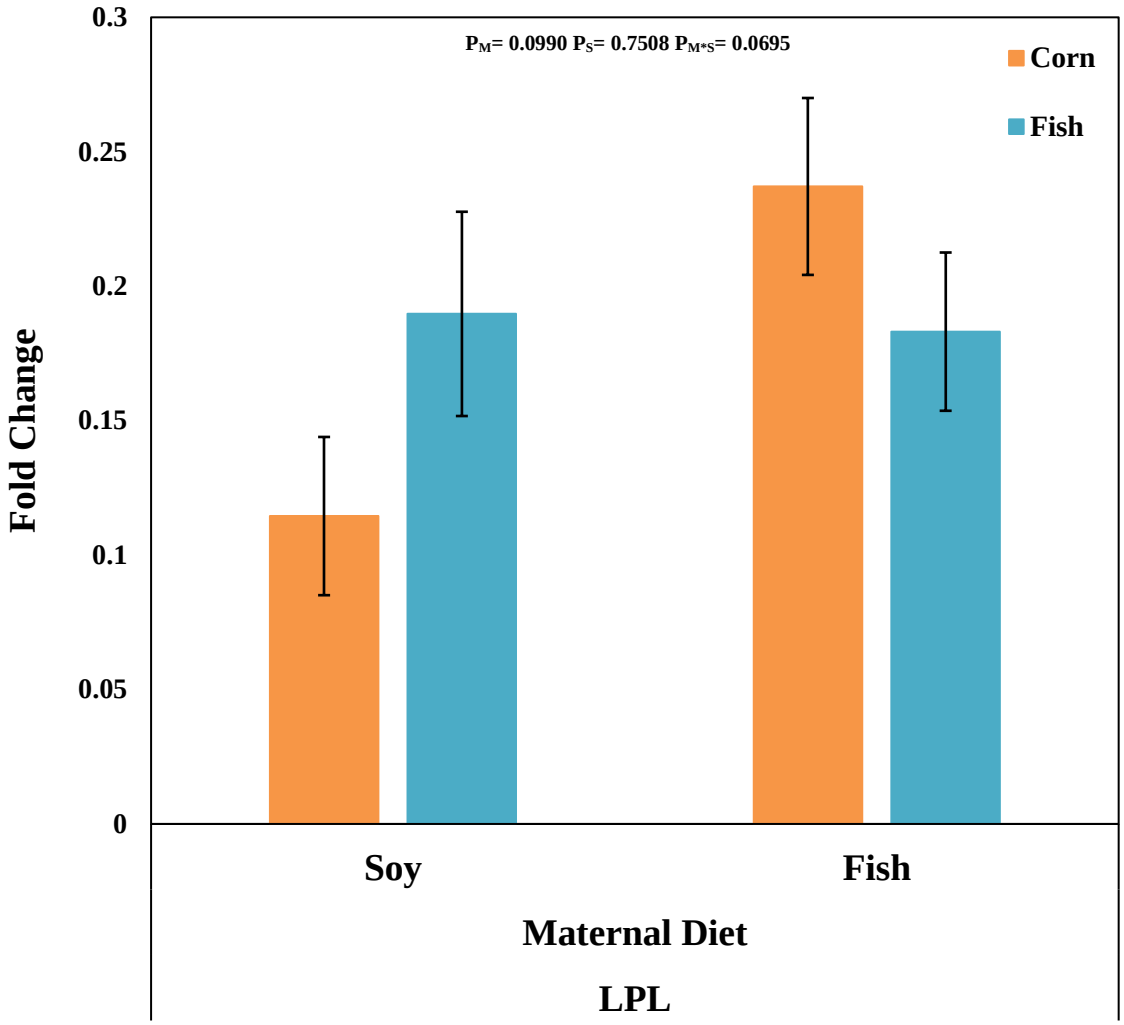

Figure 1.5 Expression of gene involved in lipid metabolism in *p.major* of chicks from SO and FO hens fed FO or CO at 14d of age. *LPL*, Lipoprotein lipase; Expression levels were normalized to expression of a reference gene (*HPRT1*) and converted to linear values using $2^{-\Delta Ct}$. Values were compared using two-way ANOVA with terms for maternal diet (P_M), starter diet (P_S) and interaction between the two diets (P_{M*S}) ($P \leq 0.05$). Values represent average relative mRNA abundance, $\pm$ SEM: FO vs. SO vs. FO vs. CO; n=3-5/diet group.

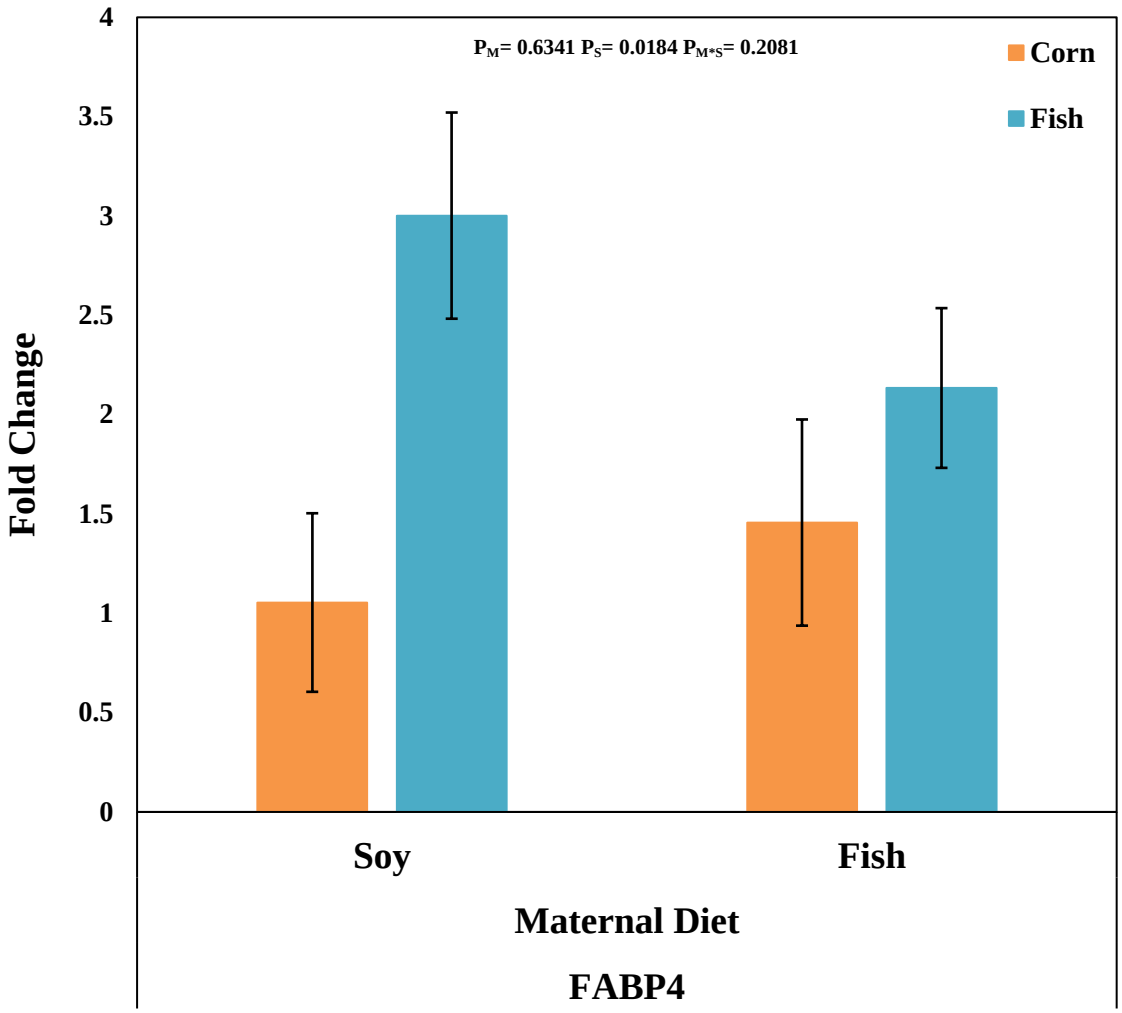

Figure 1.6 Expression of gene involved in lipid metabolism in *p.major* of chicks from SO and FO hens fed FO or CO at 14d of age. *FAPB4*, Fatty acid binding protein 4; Expression levels were normalized to expression of a reference gene (*HPRT1*) and converted to linear values using $2^{-\Delta Ct}$. Values were compared using two-way ANOVA with terms for maternal diet (P_M), starter diet (P_S) and interaction between the two diets (P_{M*S}) ($P \leq 0.05$). Values represent average relative mRNA abundance, $\pm$ SEM; FO vs. SO vs. FO vs. CO; n=3-5/diet group.

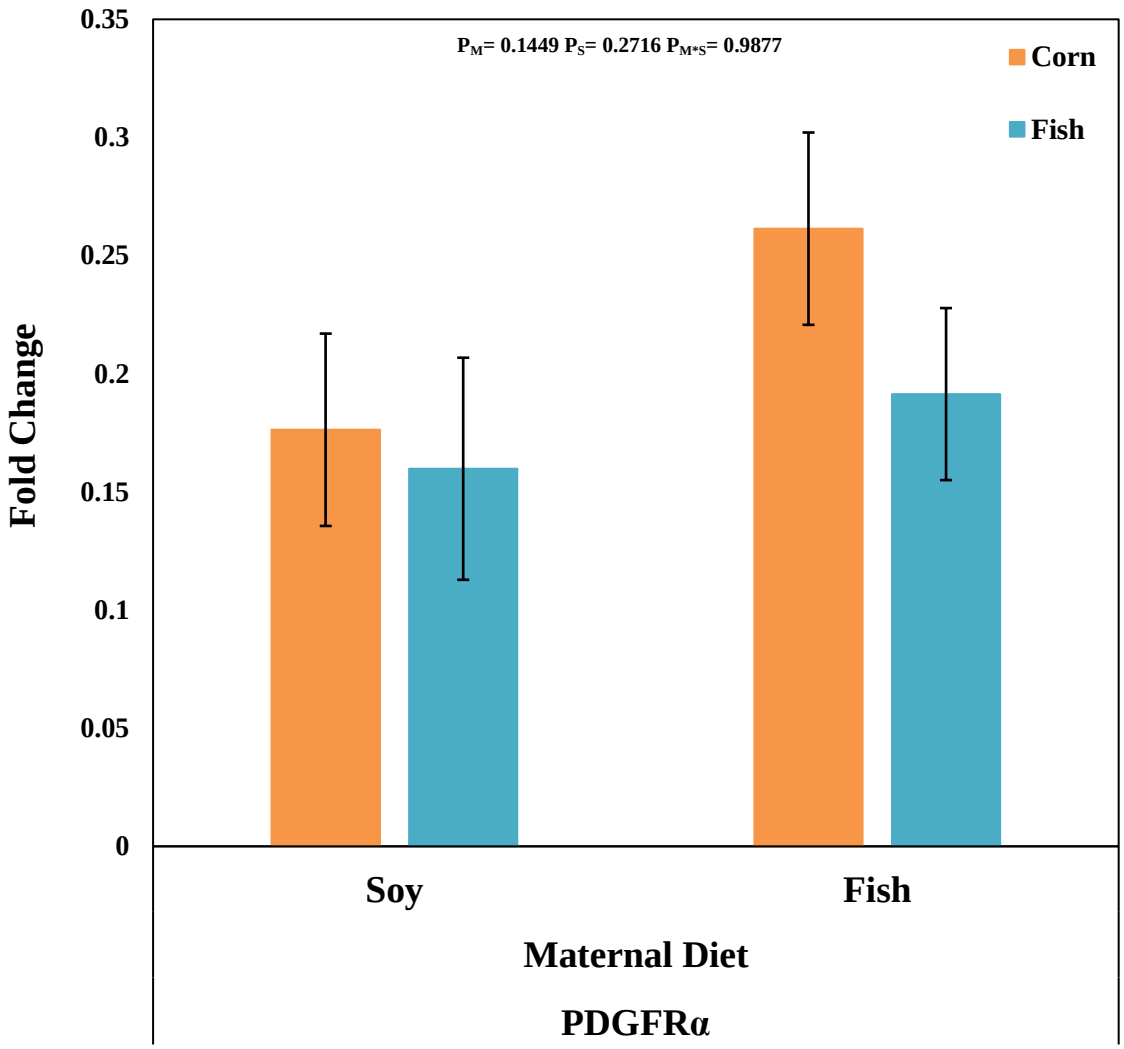

Figure 1.7 Expression of gene involved in fibroadipogenic progenitor cell activity in *p.major* of chicks from SO and FO hens fed FO or CO at 14d of age. *PDGFRα*, Platelet derived growth factor receptor alpha; Expression levels were normalized to expression of a reference gene (*HPRT1*) and converted to linear values using $2^{-\Delta Ct}$. Values were compared using two-way ANOVA with terms for maternal diet (P_M), starter diet (P_S) and interaction between the two diets (P_{M*S}) ($P \leq 0.05$). Values represent average relative mRNA abundance, $\pm$ SEM; FO vs. SO vs. FO vs. CO; n=3-5/diet group.

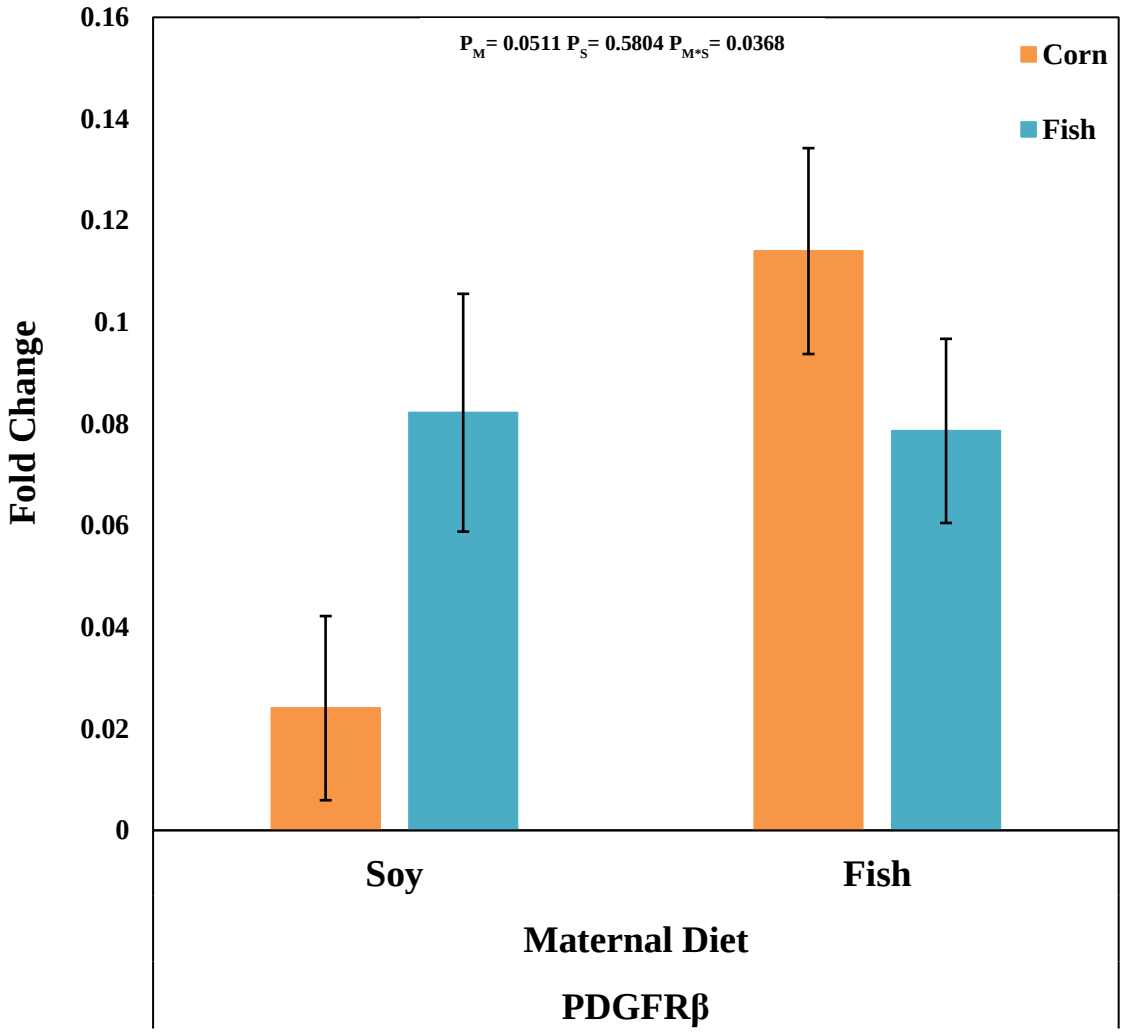

Figure 1.8 Expression of gene involved in pericyte activity in *p.major* of chicks from SO and FO hens fed FO or CO at 14d of age. *PDGFRβ*, Platelet derived growth factor receptor beta; Expression levels were normalized to expression of a reference gene (*HPRT1*) and converted to linear values using $2^{-\Delta Ct}$. Values were compared using two-way ANOVA with terms for maternal diet (P_M), starter diet (P_S) and interaction between the two diets (P_{M*S}) ($P \leq 0.05$). Values represent average relative mRNA abundance, $\pm$ SEM; FO vs. SO vs. FO vs. CO; n=3-5/diet group.

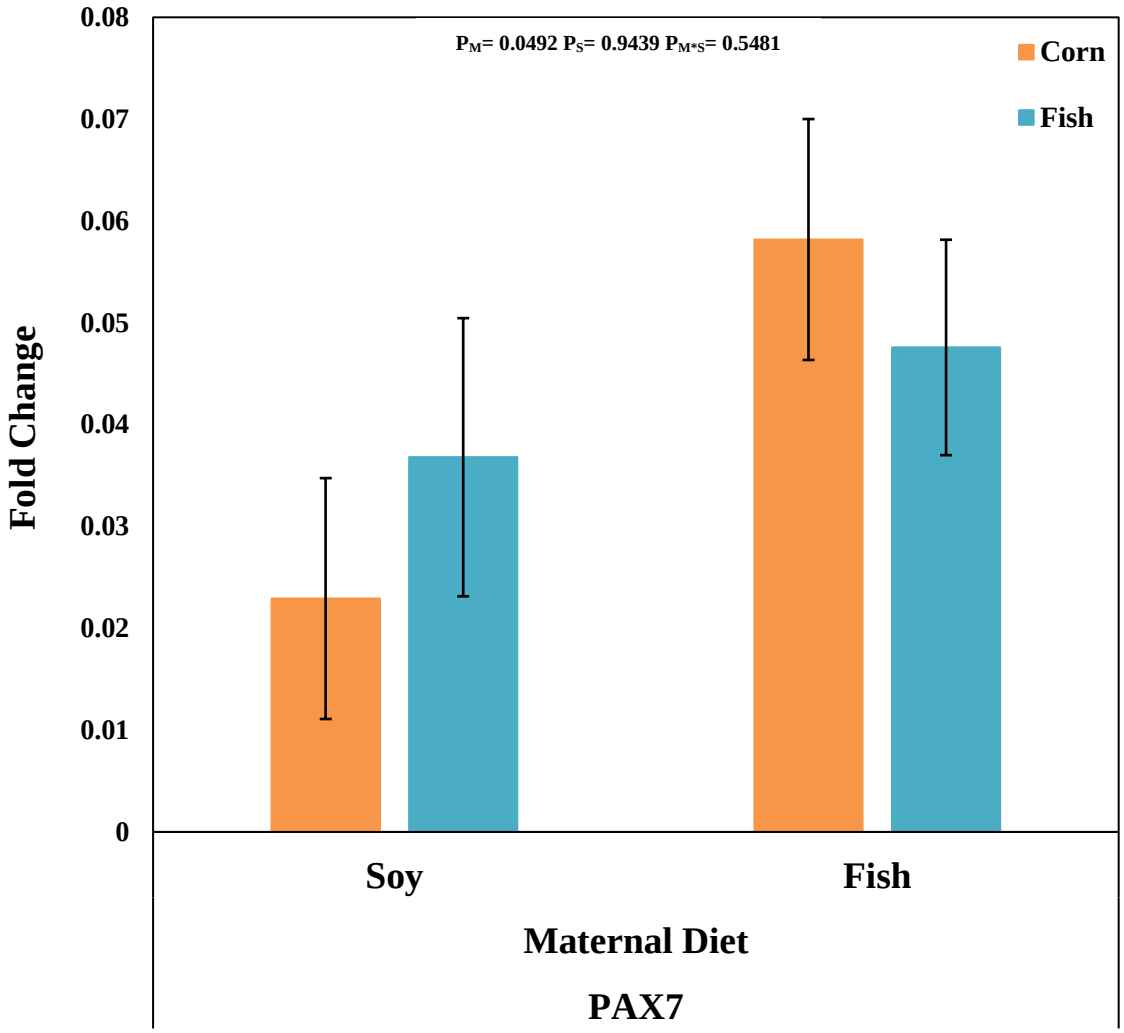

Figure 1.9 Expression of gene involved in myogenic commitment in *p.major* of chicks from SO and FO hens fed FO or CO at 14d of age. *PAX7*, Paired box 7; Expression levels were normalized to expression of a reference gene (*HPRT1*) and converted to linear values using $2^{-\Delta Ct}$. Values were compared using two-way ANOVA with terms for maternal diet (P_M), starter diet (P_S) and interaction between the two diets (P_{M*S}) ($P \leq 0.05$). Values represent average relative mRNA abundance, $\pm$ SEM; FO vs. SO vs. FO vs. CO; n=3-5/diet group.

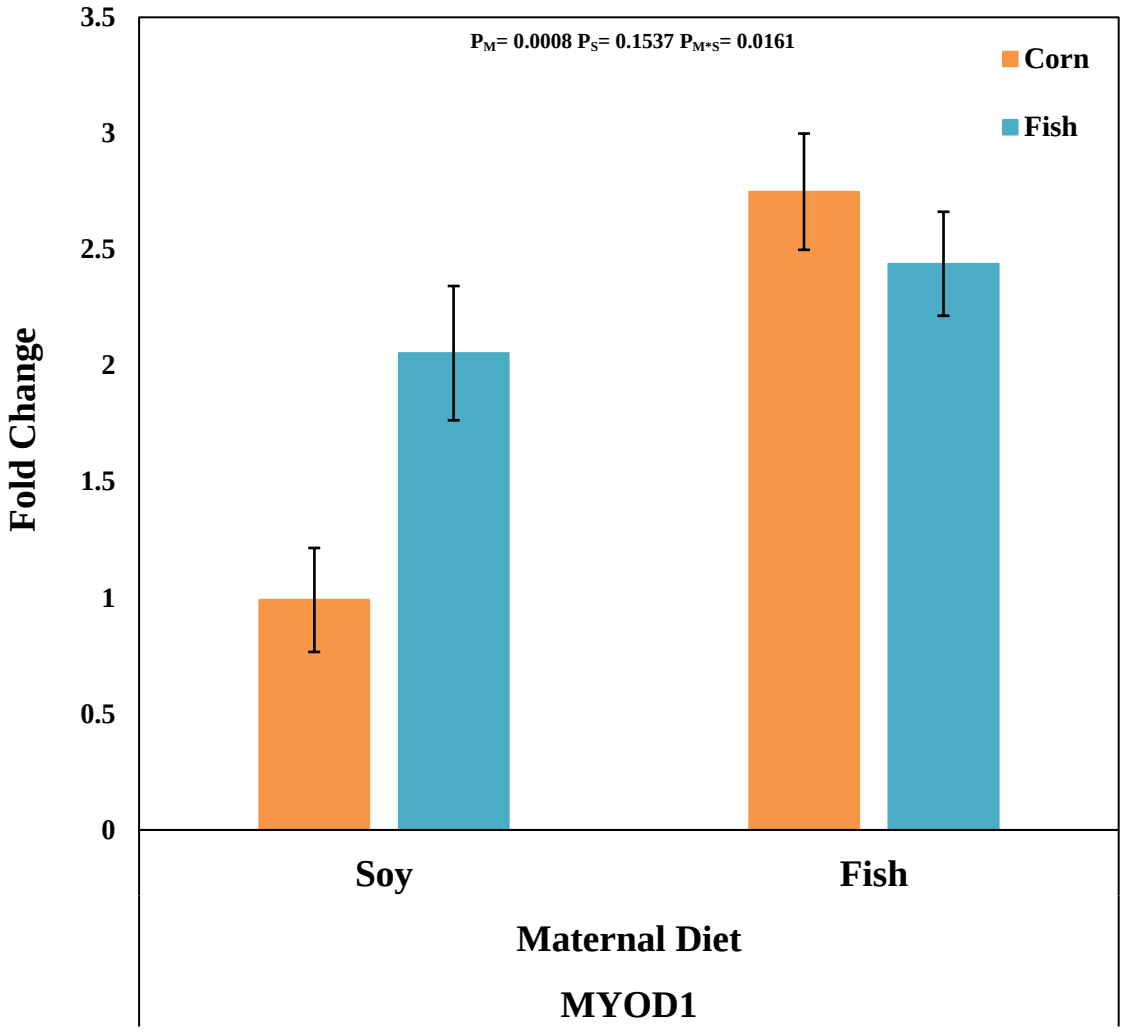

Figure 1.10 Expression of gene involved in early myogenic differentiation in *p.major* of chicks from SO and FO hens fed FO or CO at 14d of age. *MYOD1*, Myogenic differentiation 1; Expression levels were normalized to expression of a reference gene (*HPRT1*) and converted to linear values using $2^{-\Delta Ct}$. Values were compared using two-way ANOVA with terms for maternal diet (P_M), starter diet (P_S) and interaction between the two diets (P_{M*S}) ($P \leq 0.05$). Values represent average relative mRNA abundance, $\pm$ SEM; FO vs. SO vs. FO vs. CO; n=3-5/diet group.

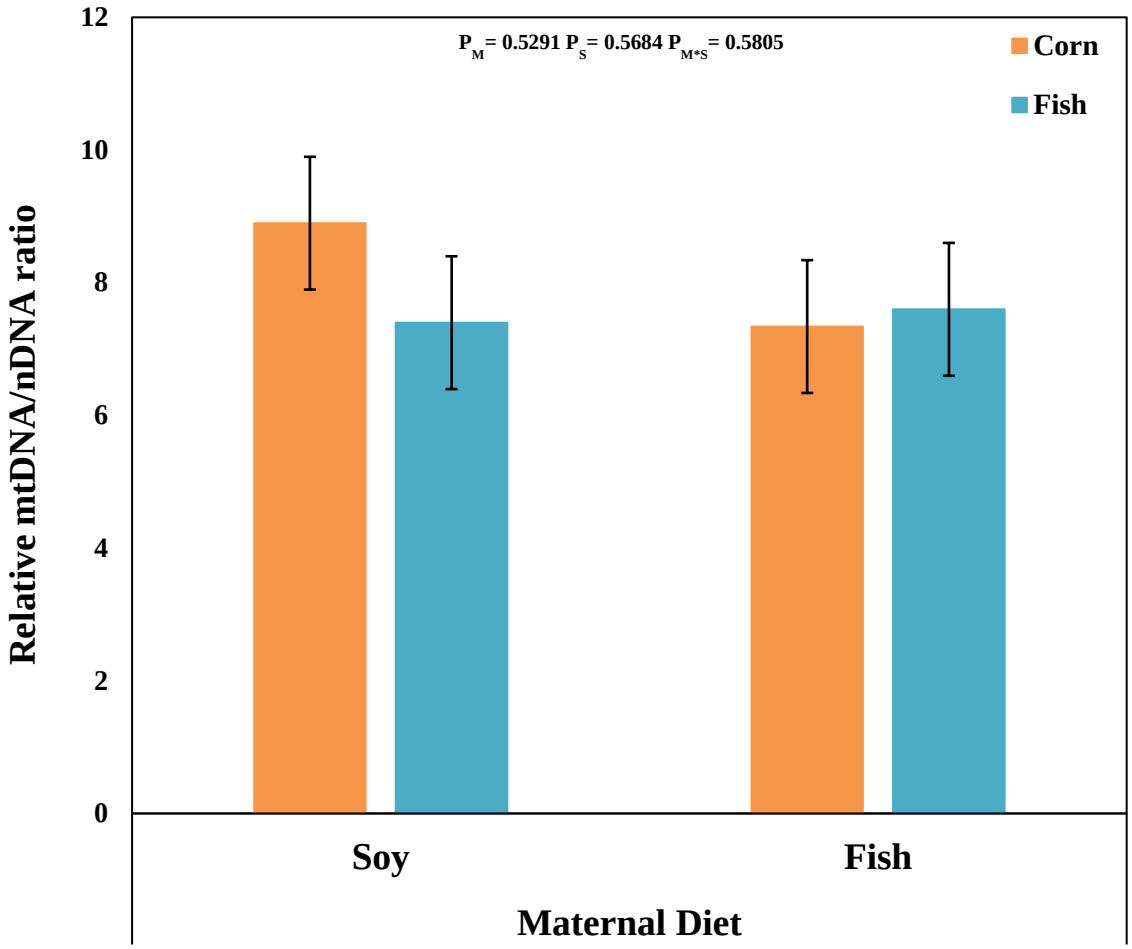

Figure 1.11 Relative mtDNA levels in *p.major* of chicks from SO and FO hens fed FO or CO at 14d of age. Measured by Cytochrome c oxidase I (*COX1*) expression normalized to expression of a nuclear reference gene (*18S*) and converted to linear values using $2^{-\Delta Ct}$. Values were compared using two-way ANOVA with terms for maternal diet (P_M), starter diet (P_S) and interaction between the two diets (P_{M*S}) ($P \leq 0.05$). Values represent average relative mRNA abundance, $\pm$ SEM; FO vs. SO vs. FO vs. CO; n=5/diet group.

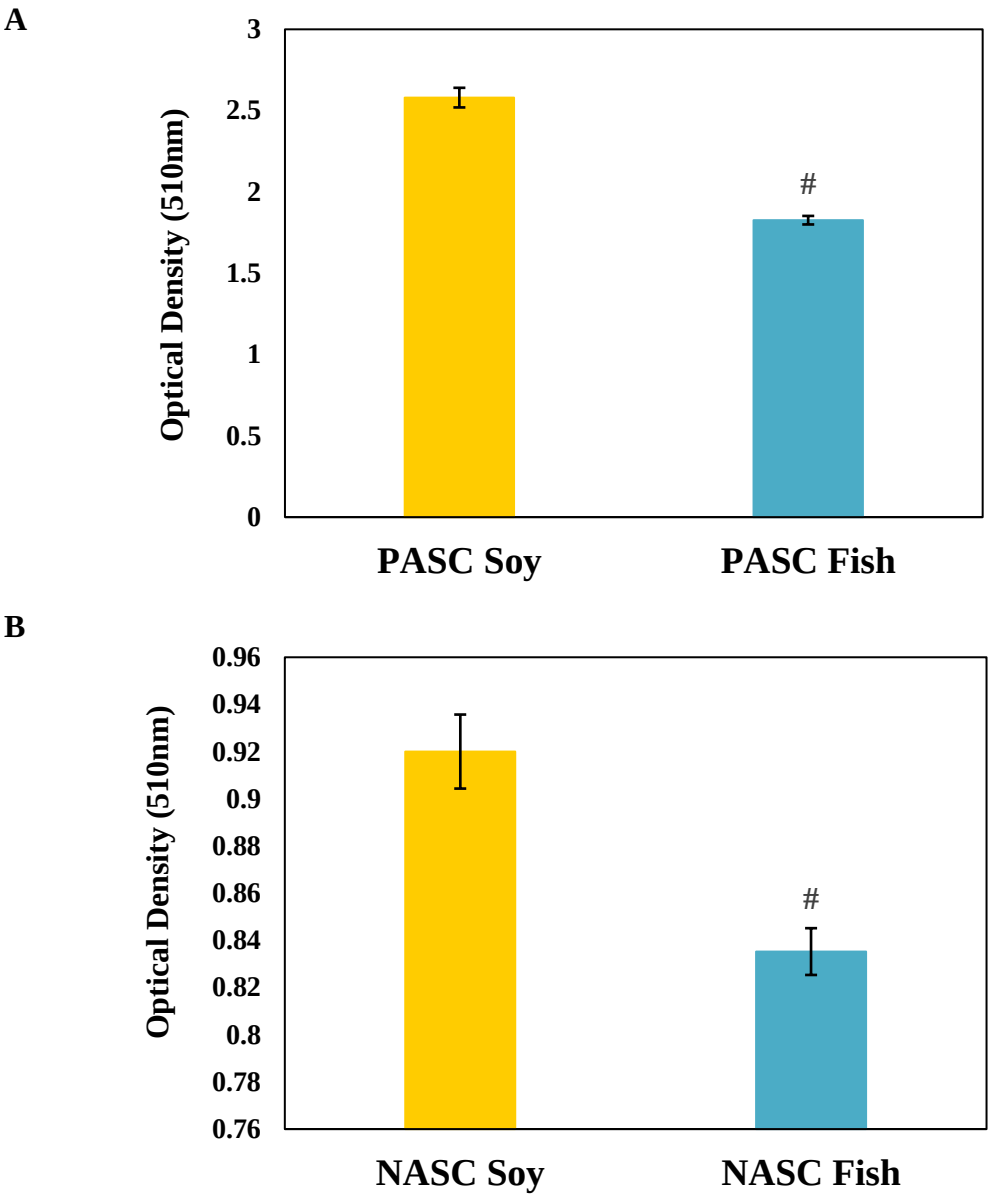

Figure 1.12 Quantification of adipogenesis of plastic adherent satellite cells (PASC) (A) and non-adherent satellite cells (NASC) (B). After capturing images the wells were de-stained with 100% isopropanol. Values were compared using a t-test. Optical density was measured at 510nm, $\pm$ SEM; # = $P \leq 0.05$, Fish vs Soy, n =24/diet

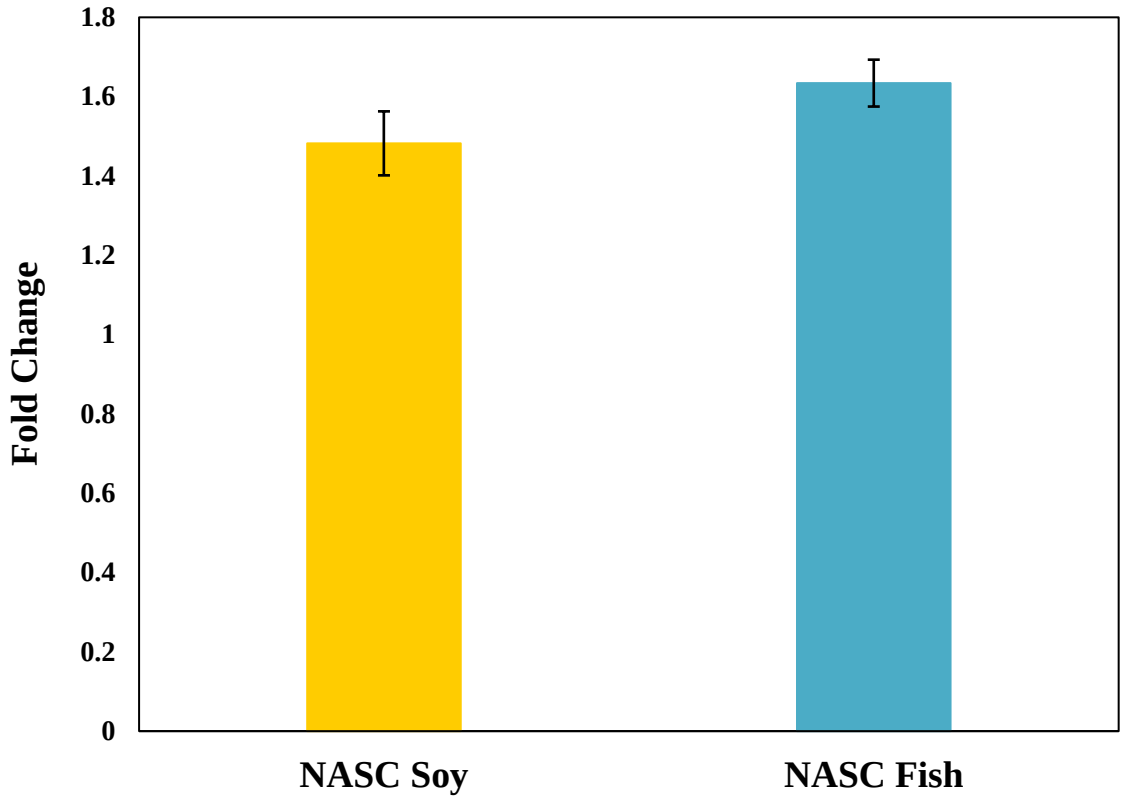

Figure 1.13 Expression of peroxisome proliferator-activated receptor gamma (*PPARγ*) in fish and soy non-adherent satellite cells. Expression levels were normalized to expression of a reference gene (*TBC*) and converted to linear values using $2^{-\Delta Ct}$. Values were compared using a t-test. Values represent average relative abundance, $\pm$ SEM; # = P $\leq$ 0.05, FO vs. SO; n=3/diet

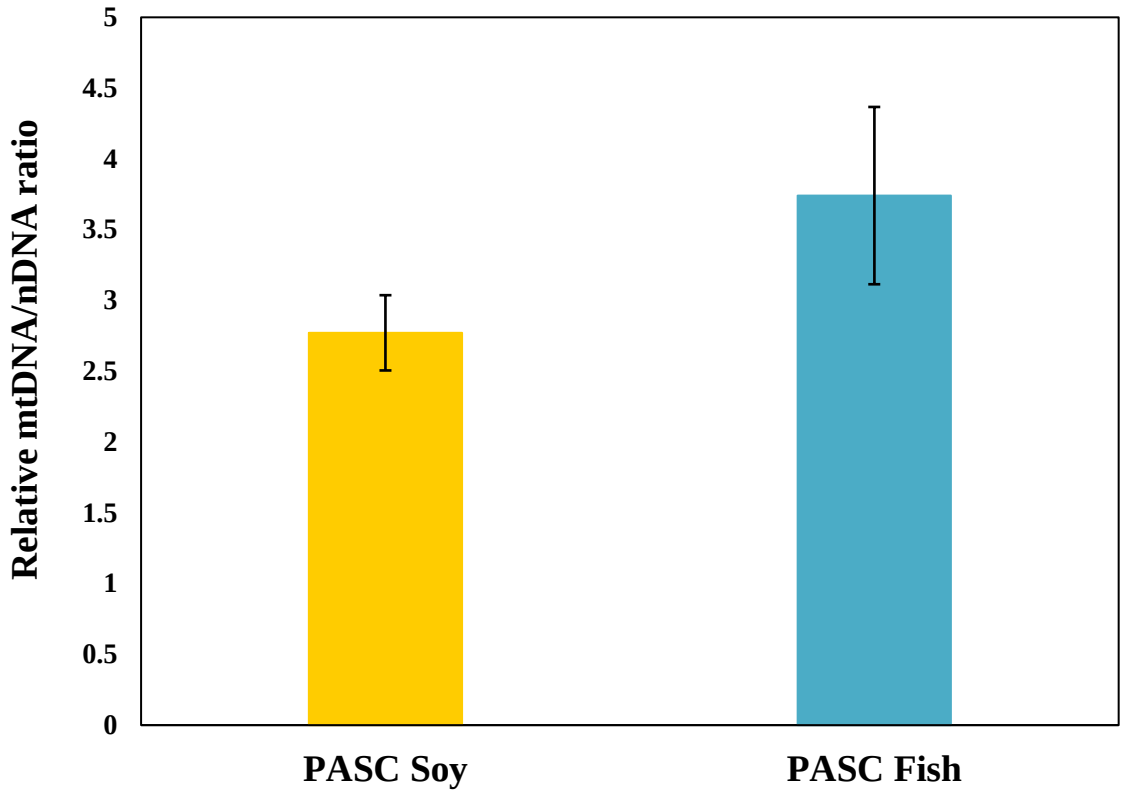

Figure 1.14 Relative mtDNA levels measured by Cytochrome c oxidase I (*COX1*) expression normalized to expression of a nuclear reference gene (*18S*) and converted to linear values using $2^{-\Delta Ct}$. Values were compared using a t-test. Values represent average relative abundance, $\pm$ SEM; # = $P \leq 0.05$, FO vs. SO; n=6/diet

Methods development: satellite cell experimental strategies

There is growing concern and limited effective strategies available to address breast muscle myopathies in the broiler industry. Wooden breast and other associated myopathies are shown to be very closely related to dysregulation of lipid metabolism and increased adipogenesis in skeletal muscle. Our lab has been successful in addressing excess adiposity in broilers at early stages in development. This prompted the exploration into addressing inappropriate adipogenesis in broilers with similar strategies. However, there are a multitude of lab specific methods to perform *in vitro* experiments with SCs isolated from chicken. This variation required us to optimize methods most suitable to effectively answer our research questions.

Isolation of SCs from *p. major* among other muscle groups varies between protocols and animals. We hybridized protocols in order to accurately remove the desired piece of tissue from *p. major* and adequately digest it to release cells from the tissue and plate on 2% gelatin to grow cells for later experimentation. However, further optimization of satellite cell purification was necessary to ensure the results of our study were accurate to that cell type. Therefore, a "pre-plating" method was instituted to further ensure that only satellite cells were being retained for experimentation. By plating frozen cells that were originally only grown on 2% gelatin on plastic tissue culture we assumed to be leaving behind any unwanted cell populations and subsequently plating remaining satellite cells in the supernatant on 2% gelatin coated dishes. However, the cell density after pre-plating varied, and it was likely that a portion of satellite cells were potentially left behind on the plastic tissue culture plate. Moving forward, we exclusively pre-plated on petri dishes and collected the supernatant after ~45 minutes of incubation. This ensured that the cells in the

supernatant we were then plating on 2% gelatin were all of the satellite cells available in the frozen vial.

Medias used for cell growth and myogenic differentiation varied as well. In chicken, satellite cells have been grown in both 10% fetal bovine serum, horse serum and chicken serum. Differentiation of satellite cells into late stage myotubes has been induced by using variations of low serum medias such as 2% or 3% horse serum or 2% horse serum with 1% bovine serum albumin. Assessing morphology and fusion behavior of satellite cells after inducing myogenic differentiation allowed us to determine the most effective myogenic differentiation induction media.

Further optimization is certainly required, but our efforts thus far have allowed us to effectively isolate and differentiate satellite cells from young broilers in *in vitro* experiments.

CONCLUSION

As more of WB syndrome's etiology is revealed it becomes more apparent that the strategy implemented to address the myopathy needs to reduce excess adipogenesis within skeletal muscle while also altering muscle fiber size hypertrophy without compromising overall growth performance. This study presented the use of the LC n-3 PUFA EPA and DHA as a tool to attenuate WB with delivery through the diet during important stages of development. The supplementation of fish oil in the perinatal diets effected the hypertrophic growth of fibers at 14 d having a greater distribution of larger fibers compared to the corn oil diet. Maternal fish oil also reduced adipocyte size significantly. Maternal fish oil also increased the expression of *PAX7*, and early marker of myogenesis potentially increasing satellite cell activity. The maternal and perinatal diets interacted to increase the expression of *MYOD1*, which is important in early myogenic differentiation, and *PDGFRβ*, potentially explaining the increase in larger muscle fibers. Expression of *FABP4* was significantly increased in chicks that consumed a diet enriched in fish oil, and while historically associated with adipogenesis, *FABP4* may be an indicator of increased fatty acid oxidation capacity in skeletal muscle. In primary SCs isolated from both maternal diets, the maternal fish oil group was found to limit triglyceride accumulation significantly. Without having grown the chicks to market age we do not know the long term impacts of fish oil on muscle morphology or adipogenesis in muscle. It would be encouraged for future studies to monitor changes in growth and adiposity from fish oil at market age to institute an effective strategy for addressing myopathies.

VITA

Kamille Ashton Piacquadio was born in Mechanicsburg, Pennsylvania on July 24, 1995. She and her immediate family remained in Pennsylvania until after her high school graduation in June of 2013 when they relocated to Myrtle Beach, South Carolina. Thereafter, Kamille attended Coastal Carolina University in Conway, SC until her graduation on May 5, 2017. She was awarded her Bachelor's of Science degree majoring in Biology and minoring in Business Administration and Chemistry. After working in the veterinary medicine field for a little over a year, she began her Master's of Science majoring in Animal Science and minoring in Nutrition at the University of Tennessee, Knoxville in August of 2018.