

**Effects of Maternal Fish Oil on Adipose Development
in the Broiler Chick Embryo**

A Dissertation Presented for the

Doctor of Philosophy

Degree

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ABSTRACT

Chapter 1 of this dissertation introduces key concepts related to adipose tissue in broiler chickens, emphasizing the role of dietary fatty acids and adipose growth mechanisms. The chapter investigates the potential of reducing fat accumulation in broiler chickens through n-3 polyunsaturated fatty acid (PUFA) supplementation. Chapter 2 presents the development and optimization of a preadipocyte isolation method from broiler chicken embryos, a crucial step that sets the stage for future investigations and experiments. Chapter 3 delves into the effects of maternal fish oil supplementation on adipose tissue development in broiler chick embryos, illuminating the influence of maternal nutrition on offspring adipose development. Chapter 4 explores the impact of maternal fish oil on the transcriptome and methylome of adipose tissue in broiler chick embryos, providing comprehensive insights into the underlying molecular mechanisms. Chapter 5 serves as a concise summary of the results and findings of this study. Collectively, this dissertation enhances our understanding of how maternal dietary n-3 PUFA can influence adipose tissue development in broiler chickens. The findings herein offer promising avenues for reducing fat accretion and enhancing the overall health of poultry, with potential implications for broader applications in animal and human health.

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CHAPTER 1 INTRODUCTION

A version of this chapter was originally published by Minjeong Kim and Brynn H. Voy:

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Abstract

Modern broiler chickens are incredibly efficient, but they accumulate more adipose tissue than is physiologically necessary due to inadvertent consequences of selection for rapid growth. Accumulation of excess adipose tissue wastes feed in birds raised for market, and it compromises well-being in broiler-breeders. Studies driven by the obesity epidemic in humans demonstrate that the fatty acid profile of the diet influences adipose tissue growth and metabolism in ways that can be manipulated to reduce fat accretion. Omega-3 polyunsaturated fatty acids (n-3 PUFA) can inhibit adipocyte differentiation, induce fatty acid oxidation, and enhance energy expenditure, all of which can counteract the accretion of excess adipose tissue. This mini-review summarizes efforts to counteract the tendency for fat accretion in broilers by enriching the diet in n-3 PUFA.

Introduction

Poultry meat is a major source of protein for much of the world. Due to population growth, rising incomes, and concerns about both health and sustainability, global demand is expected to continue to grow around the globe. Current projections are that poultry will account for 41% of all protein consumption from meat by 2030 (OECD/FAO, 2021). Broiler chickens are produced for meat, and genetic selection has produced remarkable advances in growth of broilers over the past several decades. For example, a 35d broiler weighed approximately 1.4 kg in 1985 and more than 2.4 kg in 2010, largely due to increase in muscle mass (Siegel, 2014). Inadvertently, selection of broilers

for rapid growth also increased their tendency to accumulate excess adipose tissue (Zuidhof et al., 2014). Adipose tissue in poultry is not commercially valuable, and it effectively wastes feed by allocating it away from muscle. Physiologically, the need for adipose tissue as an energy reservoir is minimal in market birds that have almost constant access to feed. In broiler-breeders, excess fat accretion also compromises fertility and welfare. The tendency to deposit excess adipose has in part been suppressed in commercial lines of broilers through refined approaches to selection (Siegel, 2014; Zuidhof et al., 2014). However, even a modest misallocation of feed, the most expensive component of production, significantly increases costs given the scale of commercial broiler production. The default approach to preventing fat accretion is feed restriction, but this has a negative impact on weight gain and meat yield and is also a welfare concern (Jackson et al., 1982; Lindholm et al., 2018).

Adipose Tissue Basics in Broilers

Chickens store energy as lipids in subcutaneous depots found near the legs and neck, and in a visceral depot in the abdomen. Subcutaneous fat is the first depot to develop and becomes visible in the embryo by embryonic day 12 (E12) (Speake et al., 1993). Adipocytes develop from a mesenchymal stem cell pool that gives rise to preadipocytes, a cell type that is committed to an adipocyte fate. Preadipocytes then differentiate and acquire a mature, lipid-storing adipocyte phenotype through a series of orchestrated changes in gene expression that are similar to those described for mammals (Rosen and MacDougald, 2006). In the embryo, development of subcutaneous fat coincides with a sharp increase in extraction of fatty acids from the yolk (Speake et al., 1998). Subcutaneous adipocytes grow rapidly as these fatty acids are taken up and stored as triacylglycerol until E19, when they are mobilized by lipolysis to provide the energy needed at

hatch (Speake et al., 1993). Abdominal fat, which becomes the main site of triglyceride storage in the mature bird, develops after hatch and becomes clearly visible around 7d of age. Adipose depots grow rapidly in broiler chicks through increases in adipocyte number (hyperplasia), as preadipocytes proliferate and undergo differentiation, and size (hypertrophy) as mature fat cells take up and store fatty acids (Bai et al., 2015). Both processes are very active during the first several weeks of life, after which hypertrophy becomes the dominant mechanism of fat deposition (Cartwright, 1991). Fatty acids that are taken up from the circulation and stored in broiler adipocytes come primarily from the liver, the main site of de novo lipogenesis in avians (Leveille et al., 1975). Fatty acids synthesized by the liver from glucose and acetate are packaged into VLDL molecules, which ferry them to adipocytes for uptake and storage. Hepatic synthesis of lipids plays a major role in adipose deposition in broilers. Divergent selection on serum VLDL level has been used to produce lines of broilers that differ significantly in fatness, illustrating the key role of the liver (Leclercq et al., 1980; Whitehead and Griffin, 1984; Baéza and Le Bihan-Duval, 2013; Resnyk et al., 2013). In addition, intrinsic differences in adipose development also contribute to excessive fat accretion in broilers. For example, significant increases in both adipocyte number and size appear as early as two weeks of age in broiler lines that have higher fat mass as they mature (Hermier et al., 1989).

Dietary Fatty Acids and Adipose Growth

Paradoxically, certain types of dietary fatty acids have been shown to inhibit accumulation of adipose tissue in a variety of species. Polyunsaturated fatty acids (PUFAs) are classified as n-3 or n-6 based on the position of the first double bond in relation to the methyl end of the molecule. Linoleic acid (LA; 18:2 n-6) and alpha linolenic acid (ALA; 18:3 n-3) are the main dietary n-6 and

n-3 fatty acids, respectively. These fatty acids are required for the formation of cell membranes but cannot be synthesized endogenously and therefore must be provided in the diet (Mukhopadhyay, 2012). Linoleic acid is enriched in a variety of common plant oils, such as those derived from corn, soybean, and safflower. Alpha linolenic acid, which is less abundant than LA, is usually obtained from flaxseed, rapeseed, and walnuts. When taken into the cell, ALA and LA can be used in formation of cell membranes, oxidized for energy, or used to synthesize longer and more highly unsaturated PUFA through a series of sequential elongation and desaturation steps (Jakobsson et al., 2006). Some of these longer chain species have unique bioactive properties through their ability to bind and activate lipid-responsive transcription factors, such as members of the PPAR family of nuclear receptors. In addition, the 20 carbon products of LA (arachidonic acid; ARA, 20:4 n-6) and ALA (eicosapentaenoic acid, EPA; 20:5 n-3) are used as substrates to produce eicosanoids, a broad family of lipid mediators that interact with various cell signaling pathways. The seemingly modest structural difference between LA and ALA has marked effects on the biochemical properties of these fatty acids and their metabolites. For example, eicosanoids produced from ARA are primarily proinflammatory, while those synthesized from EPA tend to be anti-inflammatory (Calder, 2017). The same enzymes catalyze elongation and desaturation of n-3 and n-6 PUFA. As a result, the relative synthesis of longer chain n-3 and n-6 species is determined by the ratio of n-3/n-6 in the diet.

Extensive in vivo and in vitro studies indicate that n-3 and n-6 PUFA differentially regulate adiposity in multiple species. In particular, diets enriched with n-3 PUFA are associated with reduced fat accretion (Rokling-Andersen et al., 2009; Torres-Castillo et al., 2018; Ballester et al., 2020; Chen et al., 2020a; Riera-Heredia et al., 2020). At the cellular level, n-3 PUFA have been shown to suppress adipogenesis, attenuate lipid accumulation in adipocytes, and promote an

oxidative adipocyte phenotype (Kim et al., 2006; Manickam et al., 2010; An et al., 2012; Fleckenstein-Elsen et al., 2016). Conversely, n-6 PUFAs tend to be pro-adipogenic (Gaillard et al., 1989; Negrel et al., 1989; Massiera et al., 2003). The anti-obesogenic effects of n-3 PUFA are most strongly associated with actions of EPA and docosahexaenoic acid (DHA, 22:6 n-3), the characteristic fatty acids of marine oils (Flachs et al., 2009). These two fatty acids, which are synthesized in algae and consumed by fish, influence a range of pathways that regulate adipose growth and adipocyte metabolism. Each fatty acid can bind and activate PPAR α , a nuclear receptor that transcriptionally increases fatty acid oxidation (Diep et al., 2000; Zúñiga et al., 2011). In addition, EPA and DHA activate signaling through AMPK and promote mitochondrial biogenesis, both of which augment fatty acid oxidation (Siriwardhana et al., 2012). They also increase synthesis of the insulin-sensitizing adipokine adiponectin, which further activates AMPK and amplifies energy expenditure in adipocytes (Itoh et al., 2007; Song et al., 2017). EPA and DHA are also used to synthesize several classes of lipid mediators, including eicosanoids and resolvins as well as other novel metabolites that suppress inflammation and limit metabolic stress in adipose tissue (Kuda et al., 2018). These actions indirectly oppose fat accretion by preventing inflammatory cascades that disrupt the efficient metabolism of glucose and fatty acids in adipocytes. Finally, EPA and DHA promote the browning of white adipocytes, which further enhances oxidative metabolism in adipose tissue and prevents fat accretion (Fleckenstein-Elsen et al., 2016). Browning is the process by which white adipocytes acquire the highly oxidative phenotype associated with mitochondria-rich brown fat cells. Browning includes induction of uncoupling protein 1 (UCP1), the characteristic protein of brown adipocytes, which uncouples mitochondrial respiration from ATP synthesis. These actions of EPA and DHA may not be relevant to broilers, as they are thought to lack brown adipocytes due to loss of the UCP1 gene from avian

genomes (Mezentseva et al., 2008). However, a recent study demonstrates the existence of brown-like fat cells in broiler chicks (Sotome et al., 2021). Collectively, ample evidence from multiple species suggests that dietary n-3 PUFA, particularly EPA and DHA, may be a tool for reducing fat accretion in broilers.

Reducing Fat Accretion in Broilers with n-3 Polyunsaturated Fatty Acids

Broiler diets are typically formulated with 2-5% added fat to support the intense energetic demands of rapid growth. Fat is often supplied using corn or soybean oil, both of which are readily available, cost-effective and enriched in LA. Combined with the use of corn and soybean meals as the diet base, this results in commercial diets that are heavily skewed toward high n-6/n-3 PUFA. In this context, commercial broiler diets are comparable to the modern human diet, in which high n-6/n-3 ratios have been associated with increased prevalence of obesity (Chilton et al., 2017). Considering the beneficial effects on fat accretion in other species, several groups have tested the ability to limit fat accretion in broilers by replacing a portion of LA-rich oils or saturated fat in the diet with sources enriched in n-3 PUFA. These have used a variety of experimental designs, including variation in age at which experimental diets were introduced, different amounts of added fat, and varying duration of feeding (Table 1.1). Nonetheless, they provide evidence that n-3 PUFA are also a promising tool to reduce fat accretion in broilers.

Fish and algal oils are the most direct way to increase dietary delivery of EPA and DHA to broilers. However, they are relatively expensive and more prone to lipid peroxidation than sources of ALA. Newman et al., replaced tallow (a source of saturated fat) with either fish oil or sunflower oil (as an n-6 PUFA control) in the diets of broiler chicks beginning at 3 wks of age (Newman et al., 2002). After 5 wks of feeding, fish oil significantly reduced abdominal fat pad weight and

increased the breast muscle: abdominal fat ratio in comparison to tallow controls. However, comparable effects were seen with the sunflower oil diet (Newman et al., 2002). Unlike most vertebrates, many types of algae can efficiently synthesize long-chain n-3 PUFA and are thus rich sources of EPA and DHA. Microalgae are microscopic algae species of commercial interest as renewable sources of biofuel production and animal feeds (Khan et al., 2018). Long et al. used a source of dehydrated microalgal biomass to efficiently deliver DHA in broiler diets (Long et al., 2018b). The biomass consisted of 64% fat made up primarily of palmitate (60%) and DHA (29%). Microalgae was added to the diet and 1% and 2% by weight, with the balance of fat (up to 3% total) provided by soybean oil, and chicks were fed from 1 to 42 d. Both the 1% and 2% microalgae diets significantly reduced adiposity at 42d. In addition, both diets significantly improved feed efficiency and increased weight gain, and improved antioxidant status, resulting in broader effects on performance than just reduced fatness. These results demonstrate that replacing even part of an n-6 PUFA source in the broiler diet with a DHA-rich alternative has beneficial effects on fat accretion.

Flaxseed and other oils rich in ALA provide the substrate for EPA and DHA synthesis, but the efficiency of ALA conversion to longer chain n-3 PUFA is thought to be very low in vertebrates due to catalytic properties of the elongase enzymes in the PUFA synthesis pathway (Arterburn et al., 2006; Brenna et al., 2009; Gregory et al., 2011). However, the chicken elongase enzymes (Elongation of Very Long Chain Fatty Acids-Like 2 and 5, ELOVL2 and ELOVL5) in this pathway have enhanced ability to synthesize DHA from ALA (Zuidhof et al., 2009; Gregory et al., 2013). ELOVL5 is highly expressed in broiler adipose tissue, including in the embryo (Mihelic et al., 2020). Unlike in most mammals, supplementing broiler diets with ALA significantly enriches tissues in EPA and DHA (Poureslami et al., 2010; Kartikasari et al., 2012). Accordingly,

several studies have been reported that linseed oil inclusion in broiler diet suppressed abdominal fat deposition and enhanced growth performance compared with saturated fat sources, such as tallow (Crespo and Esteve-Garcia, 2001; 2002; Ferrini et al., 2010; Gonzalez-Ortiz et al., 2013) or n-6 PUFA sources such as sunflower oil (Crespo and Esteve-Garcia, 2002; Ibrahim et al., 2018). For example, supplementing broiler diets with flaxseed oil (10%) reduced abdominal fat by 30% compared to equivalent amounts of saturated or monounsaturated fatty acids (Ferrini et al., 2008). Both fish and flaxseed oils have also been shown to reduce adipocyte size in broiler chicks, suggesting that they act relatively early on adipose development (Torchon et al., 2017). The relative effects of fish oil and flaxseed oil as replacements for sunflower oil have also been compared. Broilers were fed diets in which all or part of sunflower oil (4%) was replaced by either flaxseed or fish oil (Ibrahim et al., 2018). Each diet significantly reduced abdominal fat percentage compared to sunflower controls, while feed efficiency, gain, and antioxidant status were significantly improved. The experimental diets also improved muscle tissue weight (breast and thigh) and decreased breast intramuscular fat (IMF) content. The diets that replaced sunflower with flaxseed oil had very low levels of EPA and DHA, as did the sunflower control. However, fatty acid analyses of breast muscle further support the ability for chickens to efficiently synthesize these fatty acids from ALA. Tissue levels of EPA and DHA were significantly increased in all experimental diets, except for the lowest level (5:1, sunflower: flaxseed) of ALA inclusion.

Potential to Reduce Fat Accretion Through n-3 Polyunsaturated Fatty Acids in the Hen

Diet

Given the scale of broiler production, replacing standard fat sources with more expensive ones, such as flaxseed or fish oil, would impose a significant cost to the industry. Developmental

programming through the diet of the broiler-breeder hen is a potential alternative means to reduce fat accretion in broilers. Developmental programming refers to the ability of embryonic exposures to exert persistent effects on the physiology of offspring after birth. This concept forms the basis of the Barker hypothesis, which was proposed in 1992 to explain the influence of factors during fetal development on the risk of diseases as adults (Barker, 1992). Programming occurs in part through epigenetic mechanisms, in which pre- and perinatal factors modify the genome through methylation, chromatin acetylation, and other non-sequence-based changes. These modifications persist through cell divisions and can influence gene expression and subsequently phenotypes throughout the life of the organism as well as across multiple generations. The concept of developmental programming has been applied to a plethora of embryonic exposures, but it is rooted in the impact of the maternal diet on offspring (Barker and Osmond, 1986). Studies in mammals indicate that energy metabolism and adiposity are especially sensitive to developmental programming by the maternal diet (Holness et al., 2000; George et al., 2012; Du et al., 2013; Lukaszewski et al., 2013; Ong and Guest, 2018). For example, energy deficit in the maternal diet during pregnancy increases adiposity in offspring after birth (Howie et al., 2012). A growing body of evidence from other species suggests that n-3 PUFA can act very early in life to influence adiposity and metabolism through developmental programming. For example, higher maternal blood levels of n-3 vs. n-6 polyunsaturated fatty acids (PUFA) during pregnancy have been associated with reduced body fat and increased leanness in children (Donahue et al., 2011; Vidakovic et al., 2016a; Vidakovic et al., 2016b). Experimental studies demonstrate that the relative abundance of n-6 vs. n-3 fatty acids in the perinatal period influences adipogenesis and fat accretion. Mice born from dams with low n-6/n-3 ratios due to genetic manipulation had half the

body fat, increased energy expenditure and fatty acid oxidation, and more but smaller adipocytes as adults compared to mice born to wild type dams (Rudolph et al., 2018).

Avians are ideally suited for developmental programming by fatty acids due to the relationship between the hen diet and the yolk, and the role of yolk fatty acids in supporting the developing embryo. Approximately 60% of the yolk's dry mass consists of lipids that are transported to the egg from the hen's liver (Speake et al., 1998). This lipid supply includes triglycerides that provide the embryo with fatty acids needed for energy, and phospholipids that serve as building blocks for cell membranes. Virtually all of the triglyceride and phospholipid pools in the yolk are utilized by the time of hatch (Noble and Moore, 1964). Saturated and monounsaturated fatty acids delivered to the yolk can be synthesized in the liver through de novo lipogenesis from carbohydrate, but the PUFA components come directly from the diet. Therefore, the n-3 and n-6 profile of the yolk, and subsequently of the tissues of the developing embryo, is determined almost exclusively by the diet of the hen (Cherian and Sim, 1991; 1993; Cherian et al., 1997). This appears to be particularly true for n-3 PUFA, which are preferentially incorporated into phospholipids of the developing embryo (Speake et al., 1998). Fish oil is the most direct means to enrich eggs and developing embryos in EPA and DHA, as the hen liver directly incorporates these species into phospholipids that are packaged for delivery to the yolk. However, oils such as flaxseed can have similar effects because of the unique properties of PUFA synthesis enzymes in chicken (Cherian et al., 1997; Gregory et al., 2013). The hen liver elongates and desaturates a portion of dietary ALA before packaging for delivery to the yolk. During embryonic development, both the yolk sac and the tissues of the embryo convert additional ALA to EPA and DHA, amplifying the effects of the hen diet (Noble, 1987; Noble and Cocchi, 1990). Therefore, oils that are more affordable and more stable than fish oil could feasibly be used for dietary developmental

programming in production. Several studies have utilized the relationship between fatty acids in the hen diet and those of the embryo to demonstrate the potential for developmental programming by n-3 PUFA in chickens. For example, enriching the hen diet in different sources of n-3 PUFA has been shown to influence immunocompetence, synthesis of inflammatory mediators, bone mineralization, and antioxidant status in offspring (Hall et al., 2007; Bautista-Ortega et al., 2009; Bullock et al., 2014; Akbari Moghaddam Kakhki et al., 2020). Readers are referred to a recent review by Thanabalan and Kiarie (Thanabalan and Kiarie, 2021) for more details.

The ability to developmentally program reduced fat accretion through n-3 PUFA in the hen diet has been demonstrated in broilers. Beckford et al., fed broiler-breeder hen diets with fat provided from either corn or fish oil (2.3%) for four weeks (Beckford et al., 2017). Chicks hatched from both sets of hens were fed commercial starter diets after hatch, with no modification to the fat source. Those produced from hens fed fish oil had significantly less fat accretion at 7d and 14 d, with no impact on growth or lean tissue mass. Reduced fat accretion was associated with smaller but more abundant adipocytes, consistent with inhibition of late stage of adipogenesis. Transcriptomic and proteomic analyses of adipose tissue indicated that the hen fish oil diet inhibited pathways associated with triacylglycerol synthesis, adipogenesis, mobilization of stored fatty acids from lipid droplets, and fatty acid utilization, all of which likely contributed to reduced adipose mass. This study provides compelling evidence that the broiler hen diet is a potential avenue through which to reduce fat accretion in broilers through developmental programming by n-3 PUFA.

It is important to acknowledge the potential challenges that would result from increasing the relative content of LC n-3 PUFA in production diets. In addition to a higher cost, oils rich in long-chain PUFA species are more prone to oxidation due to increased chain length and numbers

of double bonds in their fatty acids. Supplementation with additional antioxidants, such as vitamin E, is often used to suppress oxidation, which can impair taste and meat quality. Alternative sources of LC n-3 PUFA that are naturally enriched in antioxidants may be able to offset this concern. For example, microalgae, a viable and effective alternative to fish oil as a source of EPA and DHA, have been shown to contain antioxidants that markedly reduce lipid peroxidation in broiler tissues (Tao et al., 2018).

Conclusion

Collectively, these studies demonstrate the ability to reduce the deposition of adipose tissue in broilers by enriching the diet in n-3 PUFA. In particular, the potential to limit fat accretion through developmental programming shows promise for efficiently limiting fat deposition through the diet of the hen. Strategies to continue to improve the utilization of feed for broiler production will be increasingly important as the global population continues to grow and seek affordable sources of complete protein.

Appendix

Table 1.1 Studies that investigated different dietary sources of n-3 PUFA and fat deposition in broiler chickens

Reference	n-3 PUFA source	Experimental diet	Animal (age)	Feeding period	Relevant phenotypes measured	Significant effects
Crespo and Esteve-Garcia, 2001	Linseed oil	(6% or 10%) • Tallow (T) • Olive oil (OO) • Sunflower oil (SO) • Linseed oil (LO)	Broiler chickens (21d)	22d/29d	• Growth performance • Abdominal fat pad	• ↑ feed efficiency • ↓ abdominal fat
Crespo and Esteve-Garcia, 2002	Linseed oil	(10%) • Tallow (SFA) • Sunflower oil (SO) • Linseed oil (LO)	Broiler chickens (27d)	27d	• Abdominal fat mass	• ↓ abdominal and body fat deposition in LO compared with SFA or SO
Ferrini et al., 2008	Linseed oil	(10%) • Tallow (T) • Sunflower oil rich in oleic acid (SOO) • SO rich in linoleic acid (SOL) • Linseed oil (LO) • Mix of fats (M: 55% T + 35% LO + 10% SOL)	Female broiler chickens (1d)	42d	• Abdominal fat mass	• ↓ abdominal fat % (reduced by 30%) in LO compared to saturated diet
Ferrini et al., 2010	Linseed oil	• Basal diet (BS) containing 0.5% sunflower oil (10%) • Tallow diet (TA) • Linseed diet (LO)	Female broiler chickens (1d)	36d	• Growth performance • Abdominal fat mass	• ↑ BWG • ↓ abdominal fat deposition
Gonzalez-Ortiz et al., 2013	FO and LO mixture	(10%) • Tallow diet (S) • A blend of fish oil and linseed oil (N3 diet)	Female broiler chickens (14d)	37d	• Abdominal fat mass • Adipocyte size	• ↓ abdominal fat % • ↓ adipocyte size
Torchon et al., 2017	Fish oil Flaxseed oil	(8%) • Lard (saturated) • Fish oil (FO) • Flaxseed oil • Canola oil	Broiler chickens (7d)	24d	• Growth performance • Abdominal fat mass • Adipocyte size	• ↓ adipocyte size • ↑ plasma non-esterified fatty acid levels (lipolysis)
Ibrahim et al., 2018	Fish oil Linseed oil	(4 – 4.5%) • Sunflower (C) • Fish oil (FO) • C1:FO1 • C3:FO1 • Linseed oil (LO) • C1:LO1 • C3:LO1	Broiler chickens (1d)	42d	• Growth performance • Abdominal fat mass	• ↑ BW, BWG/ ↓ FCR • ↓ abdominal fat % • ↓ TG and total cholesterol / ↑ HDL-C/ ↓ LDL-C and VLDL
Long et al., 2018	Microalgae (MA)	(3%) • Soybean oil (SO) • MA 1: SO 2 • MA 2: SO 1	Male broiler chickens (1d)	42d	• Growth performance • Abdominal fat mass	• ↑ BWG/ ↓ FCR • ↑ liver %, ↓ abdominal fat % • ↑ serum glucose/ ↓ cholesterol • ↑ superoxide dismutase, ↑ antioxidant capacity

CHAPTER 2

ISOLATION OF PREADIPOCYTES FROM BROILER CHICK EMBRYOS

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Summary

The present protocol describes a simple method for isolating preadipocytes from adipose tissue in broiler embryos. This method enables isolation with high yield, primary culture, and adipogenic differentiation of preadipocytes. Oil Red O staining and lipid/DNA stain measured the adipogenic ability of isolated cells induced with differentiation media.

Abstract

Primary preadipocytes are a valuable experimental system for understanding the molecular pathways that control adipocyte differentiation and metabolism. Chicken embryos provide the opportunity to isolate preadipocytes from the earliest stage of adipose development. This primary cell can be used to identify factors influencing preadipocyte proliferation and adipogenic differentiation, making them a valuable model for studies related to childhood obesity and control of excess fat deposition in poultry. The rapid growth of postnatal adipose tissue effectively wastes feed by allocating it away from muscle growth in broiler chickens. Therefore, methods to understand the earliest stages of adipose tissue development may provide clues to regulate this tendency and identify ways to limit adipose expansion early in life. The present study was designed to develop an efficient method for isolation, primary culture, and adipogenic differentiation of preadipocytes isolated from developing adipose tissue of commercial broiler (meat-type) chick embryos. The procedure has been optimized to yield cells with high viability (~98%) and increased

capacity to differentiate into mature adipocytes. This simple method of embryonic preadipocyte isolation, culture, and differentiation support functional analyses of fat growth and development in early life.

Introduction

Obesity is a global health threat to both adults and children. Children who are overweight or obese are approximately five times more likely to be obese as adults, placing them at significantly increased risk for cardiovascular disease, diabetes, and many other comorbidities. About 13.4% of US children aged 2–5 have obesity (Fryar et al., 2018), illustrating that the tendency to accumulate excess body fat can be set in motion very early in life. For very different reasons, the accumulation of excess adipose tissue is a concern for broiler (meat-type) chickens. Modern broilers are incredibly efficient but still accumulate more lipid than is physiologically necessary (Siegel, 2014; Zuidhof et al., 2014). This tendency begins soon after hatch and effectively wastes feed, the most expensive production component, by allocating it away from muscle growth. Therefore, for both children and broiler chickens, albeit for very different reasons, there is a need to understand factors that influence adipose tissue development and identify ways to limit adipose expansion early in life.

Adipocytes form from preadipocytes, adipose tissue-derived stem cells that undergo differentiation to develop mature, lipid-storing fat cells. Accordingly, preadipocytes *in vitro* are a valuable experimental model for obesity studies. These cells, isolated from the stromal vascular fraction of adipose depots, can provide a fundamental understanding of molecular pathways controlling adipocyte differentiation and metabolism (Poulos et al., 2010; Vilaboa et al., 2014). Chick embryos are a favorable experimental model in developmental studies because experimental

manipulation is easier by culturing eggs on the desired schedule, which enables obtaining embryos without the mother's sacrifice to observe a series of developmental stages of embryos. Moreover, complicated surgical procedures and much time are not required to obtain embryos relative to larger animal models. Therefore, the chick embryo presents an opportunity to obtain preadipocytes from the earliest stages of adipose tissue development. Subcutaneous adipose tissue becomes visible in the chick around embryonic day 12 (E12) as a clearly defined depot located around the thigh. This depot is enriched in highly proliferative preadipocytes that actively undergo differentiation under developmental cues to form mature adipocytes (Speake et al., 1993; Chen et al., 2014). The process of adipogenic differentiation is comparable between chickens and humans. Therefore, preadipocytes isolated from chick embryos can be used as a dual-purpose model for studies relevant to humans and poultry. However, the yield of preadipocytes declines with aging as cells grow into mature adipocytes (Vilaboa et al., 2014).

The present protocol optimizes the isolation of preadipocytes from adipose tissue during the stage (E16–E18) at which adipogenic differentiation and adipocyte hypertrophy are at their peak in broiler chick embryos (Farkas et al., 1996). This procedure can assess the effects of factors to which the developing embryo is exposed *in ovo*, such as the hen diet, on adipocyte development and adipogenic potential *ex vivo*. It can also test the impact of various manipulations (e.g., hypoxia, nutrient additions, pharmacological agonists, and antagonists) on adipogenesis or the various 'omes (e.g., transcriptome, metabolome, methylome) of adipocyte progenitors. As a representation of the earliest stage of adipose formation, cells obtained using this protocol are valuable models for studies relevant to poultry and humans.

Materials and methods

All animal procedures were approved by the University of Tennessee Institutional Animal Care and Use Committee. Freshly fertilized commercial broiler eggs (Cobb 500) were obtained from a local hatchery. Eggs were incubated at 38 °C with 60% relative humidity until dissections at embryonic days 16–18 (E16–E18). Adipose tissue was collected from the subcutaneous (femoral) depot.

1. Preparation for isolation and culture

1.1. Prepare the culture hood and the instruments.

1.1.1. Before starting dissections, set up a work area in the laminar-flow hood. Disinfect the working area and all the instruments by swabbing with 70% ethanol. Perform all procedures using sterile materials.

NOTE: Always swab the containers and instruments with 70% ethanol before placing them back in the cell culture hood. It is recommended to place a benchtop instrument sterilizer in the hood so that instruments can readily be sterilized between embryos.

CAUTION: When using an instrument sterilizer, cool down the hot instrument properly to prevent burn injury and tissue damage. A minimum cooling time of 3 min is recommended. Swab with 70% ethanol before use.

1.1.2. Assemble the following instruments and vessels in the hood: straight forceps (120 mm), tweezers (110 mm), two pairs of curved forceps (100 mm), two pairs of straight scissors (140 mm), curved surgical scissors (115 mm), tissue strainer (250 µm nylon mesh), cell

strainer (40 μ m nylon mesh), conical centrifuge tubes (15 mL and 50 mL), Petri dishes (60 mm and 100 mm), small beaker (100 mL), 70% ethanol spray and sterile gauze, paper towel, and benchtop wiper (see Table of Materials).

- 1.2. Prepare enzymatic solution, collection media, and culture media following the steps below.

NOTE: Prepare solutions in advance and store at 4 °C until use. Media needs to be placed on ice before tissue collection. All reagents used in this step are listed in the Table of Materials.

- 1.2.1. Prepare media used for both collections of adipose tissue and preparation of enzymatic solution by supplementing DMEM/F12 (Dulbecco's Modified Eagle Medium with 2.50 mM of L-glutamine and 15 mM of HEPES buffer) with 2.5 μ g/mL of Amphotericin B and 1x penicillin/streptomycin (P/S), 100 U/mL. Store at 4 °C until use.

NOTE: This media remains stable for 1 year when stored at 4 °C.

- 1.2.2. Prepare sterilization solution by diluting Betadine to 20% (v/v) in 1x PBS (Phosphate Buffered Saline having pH 7.4, without calcium, magnesium or phenol red) with 2.5 μ g/mL of Amphotericin B. Store at 4 °C until use.

NOTE: The solution is stable for 2 years when stored at 4 °C.

- 1.2.3. Prepare enzymatic solution by dissolving Type 1 collagenase (1 mg/mL) in DMEM/F12 with 2.5 μ g/mL of Amphotericin B and 1x P/S (step 1.2.1). Make fresh collagenase solution and maintain it on the ice during dissections. Approximately 10 min before use, prewarm by incubating this solution at 37 °C in a water bath to initiate its enzymatic activity.

NOTE: Approximately 1 mL of collagenase solution (1 mg/mL) is needed per 100 mg of adipose tissue.

- 1.2.4. Prepare growth media used for plating and propagation by supplementing DMEM/F12 media with 1x P/S and 10% FBS. Store at 4 °C until use.

NOTE: The solution is stable for 1 year when stored at 4 °C.

- 1.2.5. Prepare washing solution by supplementing 1x PBS with 2.5 µg/mL of Amphotericin B. Adjust solution to desired pH 7.4. Store at 4 °C until use.

NOTE: The solution is stable for 2 years when stored at 4 °C.

2. Adipose tissue collection and digestion

- 2.1. Euthanize the embryo.

- 2.1.1. On embryonic day 16, remove the eggs from the incubator. The primary potential source of microbial contamination is the egg's surface; therefore, swab the eggs with sterile gauze soaked in 70% ethanol prior to cracking them. After swabbing, place the egg vertically with the pointy end down onto a small beaker (100 mL) lined with paper towels to cushion the egg from the glass.

- 2.1.2. Using the forceps handle, break an egg by tapping the blunt end of the egg. Carefully remove the eggshell to create an opening sufficiently large to remove the embryo (step 2.1.3). Gently tear the white shell membrane to expose the embryo (Figure 2.1A). Pierce the amnion carefully using sterile tweezers (Figure 2.1B).

NOTE: The amniotic sac is a transparent membrane filled with amniotic fluid that encloses the embryo.

2.1.3. Remove the embryo from the egg by lightly gripping the embryo's neck using straight forceps. Sever the yolk sac to disconnect it from the embryo, and transfer the embryo to a 100 mm Petri dish.

2.1.4. Decapitate immediately using surgical scissors and forceps.

2.2. Perform the adipose tissue collection.

2.2.1. Swab the embryo body with 70% ethanol and scrub the skin surface gently with sterile gauze to remove feathers, as they can interfere with filtration at later steps after digestion. Use benchtop wipers to keep the skin and feathers from touching collected tissue.

2.2.2. Cut off the skin between the legs and abdominal region to reveal the pair of femoral adipose depots.

2.2.3. Hold the skin around the leg using curved forceps with one hand. Gently remove the femoral subcutaneous fat with the other hand, using curved forceps to gently pull the depot away from the leg.

NOTE: There is relatively little connective tissue that adheres to the fat pad to the leg, and the entire fat pad should be removable in one piece using only forceps. This can be facilitated by holding the forceps backward so that the curved portions rather than the ends clamp the fat pad for removal.

- 2.2.3.1. If necessary, cut the fat pad away with curved scissors. Repeat with the other fat pad and additional embryos as needed.

NOTE: A total of 80 mg of subcutaneous fat can be obtained from most embryos at E16 (Figure 2.1C). This typically yields $\sim 1 \times 10^6$ cells, sufficient to plate one T-25 flask. It might be useful at this step to weigh fat pads from a few embryos to assess the amount of starting material, as fat pad weights can vary across specific broiler lines, and due to uncontrollable factors, such as breeder hen age and diet. Subcutaneous fat was routinely collected from 5 eggs to ensure an adequate yield of cells for plating in multiple flasks.

- 2.2.4. Transfer the tissues into ~ 5 mL of collection media in a 15 mL tube and repeat dissection for the remaining eggs.

- 2.2.5. Briefly rinse the collected fat pads by transferring them to a 60 mm Petri dish containing sterilization solution and swirling the dish a few times.

- 2.2.6. Rinse off the sterilization solution by transferring fat pads to a 60 mm Petri dish containing 1x PBS. Swirl gently. Repeat this step by transferring to a second 60 mm Petri dish containing 1x PBS.

- 2.3. Perform enzymatic digestion and preadipocyte isolation following the steps below.

- 2.3.1. Transfer the adipose tissues to a 15 mL tube containing ~ 1 mL of pre-warmed enzymatic solution per 100 mg of tissue. Using a pair of long, straight scissors, immerse the scissors in the tube and finely mince the adipose tissues in the solution into as small pieces as possible (approx. 1 mm^3) (Figure 2.2A).

NOTE: Cell yield will be reduced if the tissues are not thoroughly minced.

2.3.2. Transfer the minced tissue and enzymatic solution to a 25 mL autoclaved flask. Wrap the flask with paraffin film. Place on an orbital shaker inside an incubator and shake at 37 °C during the digestion step.

NOTE: Alternatively, use a shaking water bath. With either approach, the speed should be sufficient to prevent pieces of tissue from settling to the bottom of the flask but must not be so fast that pieces are propelled to the top of the flask and stick to the glass, or that fluid accumulates on the paraffin film cover.

2.3.3. After ~30 min, cut the end of 1 mL pipette tip to a diameter of approximately 3 mm. Pipette the tissue mixture up and down a few times to help release the cells from the adipose tissue. Return the flask to the incubator/water bath and resume shaking.

2.3.4. After an additional 15 min, check the flask for completeness of digestion. After removing the flask from the shaker, a layer of whitish cells will form at the top of the fluid layer. If tissue fragments still remain, cut the end from another pipette tip and gently pipette the mixture up and down, then continue shaking for an additional 15 min.

NOTE: Completely digested tissue/collagenase mixture looks like milky chyme. Typically, tissue is sufficiently digested after 1 h of gentle shaking. If many fragments remain at this point, it may indicate an issue with the collagenase enzyme used. Constant shaking can increase yield. However, prolonged exposure to the enzyme and the physical stress may also damage the cells.

2.3.5. After digestion, pipette gently up and down to mix well. Filter through a 250 μ m tissue strainer into a 15 mL tube by pipetting to remove any bits of undigested tissue and debris.

2.3.5.1. Rinse the flask with 4 mL of growth media by pipetting to remove cells that may be adhered to the glass and filter into the same 15 mL tube. Rinse the strainer with additional growth media by pipetting to loosen any trapped cells, up to a total volume of 14 mL.

2.3.6. Pellet the cell fraction by centrifuging at 300 x g for 5 min at RT (7 min for 50 mL tube).

NOTE: Always swab the tubes with 70% ethanol before returning to the cell culture hood.

2.3.7. Aspirate the supernatant, being careful not to dislodge the cell pellet. Gently resuspend the pellet in 1 mL red blood cell lysis buffer (see Table of Materials) by pipetting up and down and incubate for 5 min at RT. The solution will turn reddish as red blood cells are lysed (Figure 2.2B).

NOTE: Place the RBC lysis buffer at RT before use. Chickens have nucleated red blood cells (Claver and Quaglia, 2009), and they attach to tissue culture dishes along with preadipocytes. RBC lysis buffer is used to lyse these cells to prevent their interference with accurate cell counts.

2.3.8. Add 5 mL of growth media to the tube containing the cells to dilute the lysis buffer and mix gently by pipetting up and down. Using a pipette, filter through a 40 μ m cell strainer into a new 50 mL tube and rinse the strainer with an additional 5 mL of growth media.

2.3.9. Pellet cells by centrifuging at 300 x g for 7 min at RT. Carefully aspirate the supernatant and resuspend the remaining cell pellet in 1 mL of growth media by pipetting up and down.

- 2.3.9.1. Use a hemocytometer, cell counter, and Trypan Blue stain to count cells and determine cell viability. Take 10 μ L of sample and mix with 10 μ L of Trypan Blue by pipetting. Load 10 μ L of the mixture on to hemacytometer and measure.

NOTE: Centrifugation speeds can be increased to 600 x g if sufficient cell pellets are not readily visible after the initial spin.

3. Seeding and culture of preadipocytes

- 3.1. Seed $\sim 1 \times 10^6$ cells in 4 mL pre-warmed growth media in a T-25 flask. Follow the same plating density if using other types of culture vessels. Place in tissue culture incubator and allow to attach overnight.

NOTE: Cells are cultured in a 38 °C incubator with a humidified atmosphere of 5% CO₂. The optimal temperature for the growth of avian cells (Nema and Khare, 2012) is 38 °C, and they grow slowly at 37 °C.

- 3.2. Next day, aspirate media and gently wash the cells with 1x PBS by pipetting to remove unattached or dead cells. Replace with 4 mL of fresh growth media. Check the cells under a microscope. Cells should be spindly shaped, like fibroblasts (Figure 2.2A).

NOTE: Typically, preadipocytes attach fairly quickly (within a few hours). The success or failure of cell isolation can be confirmed at this time (after 24 h).

- 3.3. Replace with 4 mL of fresh growth media every 2 days and subculture or cryopreserve cells when they reach 70% - 80% confluence (Figure 2.3C).

4. Subculturing and cryopreservation

- 4.1. Aspirate old media and gently wash cells with 4 mL of 1x PBS by pipetting. Aspirate PBS, add 2 mL of 0.1% trypsin to cover the cell surface in a T-25 flask (adjust the volume accordingly for other culture vessels), and then incubate for 3–4 min at 38 °C.

NOTE: Observe if the cells are detached from the culture plate. Tap the culture plate gently to help cell detachment. Incubating cells with trypsin for too long will damage cells.

- 4.2. Add an equivalent volume of pre-warmed growth media to inhibit the trypsin reaction. Several times, pipette over the cell surface, tilting the plate to loosen the remaining cells.
- 4.3. Transfer the cell suspension to a 15 mL tube and centrifuge at 300 x g for 5 min at RT (7 min for 50 mL tube). Aspirate supernatant.
- 4.4. If subculturing, resuspend pellet in 1 mL growth media by pipetting up and down. Count cells as described in step 2.3.9.1 and then replate using the same plating density used initially in step 3.1.
- 4.5. If cryopreserving, prepare 4 mL of freezing media for a T-25 flask with 90% confluency.

NOTE: Freezing media consists of 10% DMSO, 30% FBS, and 60% DMEM/F12.

- 4.6. Resuspend cell pellet in freezing media and transfer 1 mL of freezing media into a cryovial. Freeze the cryovials slowly to -1 °C/min using a freezing container. Place the container in a -80 °C freezer overnight and then transfer it to liquid nitrogen for long-term storage.

NOTE: Properly cryopreserved preadipocytes maintain their viability for at least three years and typically perform like freshly isolated cells when thawed and plated.

5. Adipogenic differentiation

NOTE: 2% gelatin-coated plates can be used to enhance cell adhesion.

- 5.1. Prepare a 2% (w/v) solution by dissolving gelatin (see Table of Materials) in distilled water. Sterilize by autoclaving at 121 °C, 15 psi for 30 min. Coat culture surface with 5–10 μ L of gelatin solution/cm² (i.e., 100–200 μ g/cm²). Swirl gently to allow evenly coat the surface.
- 5.2. Inspect plates to ensure even spreading of the gelatin solution as some regions may remain uncoated initially. Allow the gelatin-coated plate to remain at RT for at least 1 h. Remove the entire volume of gelatin solution from the wells.

NOTE: This will leave a thin gelatin coat at the bottom of the wells/dishes. Gelatin solution can be reused several times (at least ten times) without affecting cell adhesion and growth. Let the gelatin-coated dishes sit in the tissue culture hood for at least 30 min before plating the cells.

- 5.3. Induce the chicken preadipocytes to undergo adipogenic differentiation by supplementing growth media with fatty acids. To prepare adipogenic differentiation media (ADM), supplement DMEM/F12 with 10% chicken serum, 1x Linoleic Acid-Oleic Acid-Albumin (9.4 μ g/mL), and 1x P/S (see Table of Materials). Make ADM fresh and warm before use.

NOTE: Chicken preadipocytes are commonly induced to undergo adipogenic differentiation by supplementing media with fatty acids rather than hormonal cocktails typically used for adipocytes from other species (Regassa and Kim, 2013).

- 5.4. Induce differentiation by replacing growth media with adipogenic differentiation media when cells reach ~90% confluence. Maintain cells in this media, replacing them every two days. Assess the differentiation visually under a microscope (20x) based on the formation of lipid droplets, which become readily visible within 48 h of inducing differentiation.

6. Assessing adipogenesis

- 6.1. Perform Oil Red O staining following the steps below.
 - 6.1.1. Plate the cells in a 6-well plate and induce adipogenic differentiation at ~90% confluency.
 - 6.1.2. Prepare a working solution of Oil Red O by combining six parts of the Oil Red O stock solution with four parts of distilled water in a 50 mL tube. Gently mix by pipetting up and down and let stand for 10 min at RT, and then filter through a Grade 1 filter paper (see Table of Materials) inside the funnel by slowly pouring the solution into a 50 mL tube.

NOTE: Oil Red O stock solution is made by dissolving 0.7 g of Oil Red O (see Table of Materials) in 200 mL 100% isopropanol in an autoclaved bottle. Mix well and let it sit for 20 min. Store at 4 °C and keep away from light until use. This is stable for one year. The working solution can be used for 3 h; however, it is recommended to use it within 2 h.

- 6.1.3. Remove media and gently wash the wells twice with 2 mL of pre-warmed 1x PBS using a pipette. Remove PBS completely by pipetting off. Fix cells with 2 mL of 10% buffered

formalin and wrap the plate with paraffin film. Leave at RT for at least 1 h and up to 2 days before staining.

NOTE: To make 1 L of 10% buffered formalin solution, mix 100 mL of 37% formaldehyde, 4.09 g of NaH_2PO_4 , 6.5 g of Na_2HPO_4 (see Table of Materials), and 900 mL distilled water. Do not pipette formalin directly onto the cells. Dispense gently on the sidewall near the bottom of the well.

6.1.4. Remove formalin and gently wash the wells with 2 mL of distilled water. Replace with 2 mL of 60% isopropanol. After 5 min, remove isopropanol and let the wells dry completely for about 10 min. Perform all staining steps at RT.

6.1.5. Add 1 mL of Oil Red O working solution to cells and incubate for 10–20 min at RT. Remove stain by pipetting off and wash five times by dipping in tap water until no excess stain is seen. After the final rinse, add 1 mL of water to cells prior to visualizing staining and collecting images under a microscope.

6.1.6. To quantify lipid accumulation per dish, remove water and extract Oil Red O dye from cells by adding a 1–2 mL of 100% isopropanol that is sufficient to cover cells in a well of 6-well plate completely. Incubate with gentle shaking on a plate shaker for 10 min at RT.

6.1.7. Transfer 200 μL of the extraction into a well of the 96-well assay plate. Quantify the relative amount of stain using a spectrophotometer plate reader to measure absorbance at 495 nm.

6.2. Perform the staining assay of the intracellular lipid droplets and the nucleus.

6.2.1. Plate cells in black bottom 96-well plates and induce adipogenic differentiation as described in step 5.3.

6.2.2. To stain the cells, add 200 μ L of the staining solution containing a fluorescent lipid stain and a fluorescent DNA stain (see Table of Materials) in 1x PBS per well. After calculating the total volume of the stain required, add 2 drops of the DNA stain and 25 μ L of the lipid stain per mL of pre-warmed 1x PBS using a foil-wrapped tube to protect the solution from light. Incubate at RT for 20 min and protected from light.

6.2.3. Read the fluorescence using a fluorescent plate reader (see Table of Materials). Detect the lipid stain (Excitation: 485 nm/Emission: 572 nm) using a red filter and the DNA stain (Excitation: 359 nm/Emission: 450 nm) through a blue/cyan filter.

NOTE: Staining can also be visualized and images captured under a fluorescent microscope.

6.2.4. Normalize the lipid stain intensities to the DNA stain intensities to quantify lipid accumulation relative to cell number (Temkin et al., 2016).

Representative results

Primary preadipocytes are morphologically similar to fibroblasts, with irregular, star-like shapes and a central nucleus (Figure 2.2A–C). The cells readily adhere to tissue culture plastic and begin to proliferate soon after attachment. They rapidly differentiate and accumulate lipid droplets (Figure 2.3D) when provided with fatty acids in the media. The viability (98%, based on dye exclusion) reported in the isolations represented here is typical. While the cells are robust aggressive handling during isolation leads to cell damage (Figure 2.3E), yielding cells that attach

poorly and fail to proliferate. Despite the procedures incorporated to prevent microbial contamination, transfer of non-sterile material can occur (Figure 2.3F). Because of their rapid growth rate, chick embryo preadipocytes consume glucose in the media at high rates. Media should be changed every 48 h to maintain their supply of energy.

The representative results presented here illustrate the adipogenic potential of chick embryo preadipocytes. These cells quickly accumulate to develop lipid droplets under adipogenic conditions, and accumulation progresses over time (Figure 2.4–5). Two methods have been presented which can be used to visualize better and quantify the degree of lipid accumulation in these cells, which is a direct reflection of adipogenesis. Staining lipids with Oil Red O is a low-cost method to visualize and quantify the accumulation of lipid droplets (Figure 2.4). A light microscope is used to collect images, and stained cells can be held on the benchtop until images are collected. Lipid accumulation in each dish of cells can be quantified as described by extracting the stain and reading the absorbance at 495 nm using a spectrophotometer. Using the combination of the selected lipid and DNA stains, it was possible to quantify the lipid accumulation relative to cell number, which compensates for non-adipogenic cells that may persist in culture (Figure 2.5). If measures are taken over several time points (Figure 2.5B and C), this combination makes it possible to assess both adipogenesis and proliferation, for example in response to added hormones or peptides.

Discussion

Although several well-described protocols have reported the isolation of preadipocytes (Hausman et al., 2008; Lee et al., 2012; Church et al., 2014; Akbar et al., 2021), isolation for embryonic preadipocytes has been optimized, which can be used for functional analyses of early life fat

growth and development in broiler chicks. This protocol yields high viability embryonic adipocyte progenitors with high differentiation potential. Moreover, the presented procedure for isolating preadipocytes is not limited to embryos but can be used in post-hatch chicks. However, it has been optimized for use with E16 embryos, and yields from chicks after hatch are considerably lower (Table 2.2), likely due to increases in the relative amount of connective tissue as chicks grow rapidly.

The success of cell isolation is ultimately evaluated by observing the shape and number of attached cells (Figure 2.3A). Low cell number or damaged cells can be observed when the tissue fractions are digested too long, especially when tissue dissociation progresses for more than 1.5 h (Figure 2.3E). Therefore, it is recommended that 1.5 h of digestion is not exceeded. On the other hand, if the tissue fragment remains clearly after an hour of digestion, the digestive time or the amount should be increased. The obtained adipose tissue amount also might be varied depending on the genetic strain of the chicken. If this protocol is used to isolate cells of other ages or species, both the amount of collagenase and digestion time will likely need to be modified.

A common limitation of primary cell culture, isolated cell begins to lose adipogenic potential after several passages in culture; thus, it is important to induce differentiation within a few days of isolation to ensure the embryonic preadipocytes do not lose their adipogenic ability. Adipogenic precursors of embryos readily differentiate into mature adipocytes in the media formulation described above, without the need for confluence or hormonal induction. The cells up to passage 4 (10–14 days) are easily differentiated within 48 h inducement (Figure 2.3D).

Controlling cell contamination in primary cell culture is another challenge, where fungal contamination is the most common. The use of Amphotericin B, as described, is generally effective at preventing fungal growth (Perlman et al., 1961; Kuhlmann, 1995). It can be included at low

concentrations in the culture media with no noticeable effects on viability or adipogenic potential. Unidentifiable microbial contaminants have been observed in the form of black swimming dots appearing a few days after cell isolation (Figure 2.3F). These adversely affected cell growth and were impossible to remove once this infection occurred (Yang et al., 2018). Whether these contaminants were an unknown microbial species found in or on eggs or arose from another source is unknown. This protocol attempts to minimize the risk of contamination by extracting embryos aseptically from the egg (Freshney, 2015). Using a heat-based instrument sterilizer in the hood during dissection also helps to minimize the potential for contamination, especially when multiple embryos are dissected.

One consideration in the process is decreased cell adhesion to tissue culture plate during differentiation, resulting from physical changes as cells form and expand lipid droplets. Although contained within the cells, lipid droplets are buoyant and, when large, appear to act as balloons that tend to promote cells lifting from the surface. Thus, care should be taken in handling preadipocytes while inducing differentiation, and particularly when adipogenesis is assessed. While the protocol presented here does not modify the culture surface, cell adhesion can be enhanced when plating cells on dishes coated with 2% gelatin. It is also important to confirm the pH of wash solutions to be 7.4 and to use pre-warmed PBS solution when washing cells and mixing with the DNA stain to reduce cold stress.

Using multiple embryos, sufficient numbers of cells can be isolated to yield experimental replicates without the need for multiple passages, expansion and the risk of cells losing their adipogenic potential. RNA can be easily isolated from both pre-and differentiated chick embryo adipocytes isolated using phenol- or membrane-based commercial methods for gene expression

studies. Sufficient RNA can be obtained from a single well of a 6-well plate for use in cDNA synthesis and follow-on qPCR or RNAseq.

In summary, the accessibility of adipose depots in the chick embryo to isolate a cell model that is highly relevant for both poultry and humans. This protocol is relatively simple to perform, and it yields a high percentage of viable cells that can be readily induced to undergo adipogenic differentiation in vitro. Fertilized chicken eggs can be obtained from various commercial sources for minimal cost, making these cells a readily available model for practical use.

Appendix

Table 2.1 Materials

Name	Company	Catalog Number	Comments
1 mL Pipette	Eppendorf	Z683825	Single Channel Pipette, 100 - 1000µL
1 mL Pipette Tip	Fisher Scientific	02-707-402	
100% Isopropanol	Fisher Scientific	A426P4	
1x PBS	Gibco	10010023	
25 mL Flask	Pyrex	4980-25	
37% Formaldehyde	Fisher Scientific	F75P-1GAL	
6-Well Plate	Falcon	353046	Tissue Culture-treated
96-Well Assay Plate	Costar	3632	
96-Well Plate, Black Bottom	Costar	3603	Tissue Culture-treated
AdipoRed	Lonza	PT-7009	
Amphotericin B	Gibco	15290026	
Bench Top Wiper (Kimtechwiper)	Kimberly-Clark	34155	
Betadine	Up & Up	NDC 1167300334	20% Working Solution
Cell Counter	Corning	6749	
Cell Strainer, 40 µm	SPL	93040	
Centrifugaton	Eppendorf	5702	
Chicken Serum	Gibco	16110082	
Conical Centrifuge Tubes, 15 mL	VWR	10025-690	
Conical Centrifuge Tubes, 50 mL	Falcon	352098	
Cryovial	Nunc	343958	
Curved Forceps, 100 mm	Roboz Surgical	RS-5137	
Curved Surgical Scissors, 115 mm	Roboz Surgical	RS-6839	
Distilled Water	Millipore	SYNSV0000	Dispensed as needed
DMEM/F12	HyClone	SH30023.01	
DMSO	Sigma	D2650	
Ethanol	Decon Labs	2701	70% Working Solution
Fetal Bovine Serum (FBS)	Gibco	10437028	
Fluorescent Microscope	EVOS	M7000	
Fluorescent Plate Reader	Biotech	Synergy H1	
Foil	Reynolds		Heavy Duty Aluminum Foil, 125 SQ. FT.
Freezing Container	Thermo Scientific	5100-0001	
Gelatin	Millipore	4055	2% Working Solution
Hematocytometer	Corning	480200	0.1 mm deep
Incubator	Fisher Scientific	6845	
Instrument Sterilizer	VWR	B1205	
Linoleic Acid-Oleic Acid-Albumin	Sigma	L9655	1x Working Solution
Microscope	Evos	AMEX1000	

Table 2.1 continued

Name	Company	Catalog Number	Comments
Multi-Channel Pipette	Thermo Scientific	4661070	12-Channel Pipettors, 30 - 300 µL
Na ₂ HPO ₄	Sigma	S-7907	
NaH ₂ PO ₄	Sigma	S-3139	
NucBlue	Invitrogen	R37605	
Oil Red O	Sigma	O-0625	
Orbital Shaker	IKA	KS130BS1	
Paper Towel	Tork	RK8002	
Parafilm	Parafilm M	PM996	
Penicillin/Steptomycin (P/S)	Gibco	15140122	1x Working Solution
Petri dishes, 100 mm	Falcon	351029	
Petri dishes, 60 mm	Falcon	351007	
Plate Shaker	VWR	200	
RBC Lysis Buffer	Roche	11814389001	
Reagent Reservior	VWR	89094-680	
Small Beaker, 100 mL	Pyrex	1000-100	
Spectrophotometer Plate Reader	Biotek	Synergy H1	
Sterile Gauze	McKesson	762703	
Straight Forceps, 120 mm	Roboz Surgical	RS-4960	
Straight Scissors, 140 mm	Roboz Surgical	RS-6762	
T-25 Flask	Corning	430639	Tissue Culture-treated
Tissue Culture Incubator	Thermo Scientific	50144906	
Tissue Strainer, 250 µm	Pierce	87791	
Trypan Blue Stain	Gibco	15250061	
Trypsin	Gibco	15400054	0.1% Working Solution
Tweezers, 110 mm	Roboz Surgical	RS-5035	
Type 1 Collagenase	Gibco	17100017	
Water Bath	Fisher Scientific	15-462-10	
Whatman Grade 1 Filter Paper	Whatman	1001-110	

Table 2.2 Average cell number and viability of isolated cells from embryo and post-hatch chicks

Age (n=)	x 10⁶ cells/100 mg tissue	Viability, %
E12 (4)	0.97 ± 0.115 ^{ab}	98.5 ± 0.58 ^a
E14 (4)	1.22 ± 0.232 ^{ab}	98.3 ± 0.96 ^{ab}
E16 (21)	1.61 ± 1.717 ^a	97.6 ± 1.58 ^a
E17 (4)	0.81 ± 0.282 ^{ab}	96.8 ± 2.63 ^{ab}
E18 (7)	0.72 ± 0.611 ^{ab}	95.9 ± 1.81 ^{ab}
E20 (4)	0.94 ± 0.171 ^{ab}	97.8 ± 0.8 ^{ab}
D4 (9)	0.24 ± 0.164 ^{ab}	93.6 ± 4.28 ^b
D5 (4)	0.25 ± 0.073 ^{ab}	98.5 ± 0.71 ^{ab}
D7 (10)	0.17 ± 0.162 ^b	96.8 ± 3.49 ^{ab}
D14 (4)	0.25 ± 0.051 ^{ab}	99.0 ± 0.00 ^{ab}

Values are expressed as mean ± SD. ^{a,b} $P < 0.05$ by one-way ANOVA with Tukey's HSD test.



Figure 2.1 Adipose tissue collection

(A) On breaking the eggshell, the white shell membrane was revealed. (B) Piercing the amnion using sterile tweezers. (C) A total of ~80 mg of femoral subcutaneous fat can be obtained from E16.

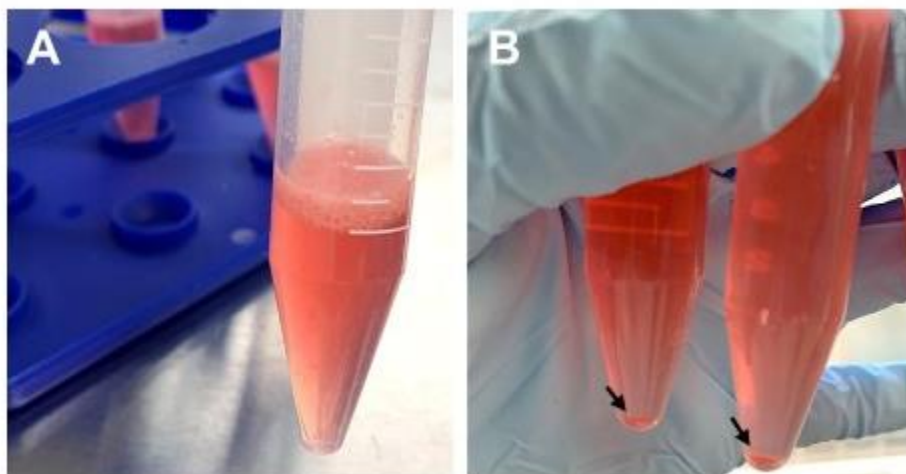


Figure 2.2 Tissue fragments for enzymatic digestion and cell pellet after digestion

(A) Minced adipose tissue in enzymatic solution (~1 mm³). (B) Arrows indicate cell pellets after RBC lysis.

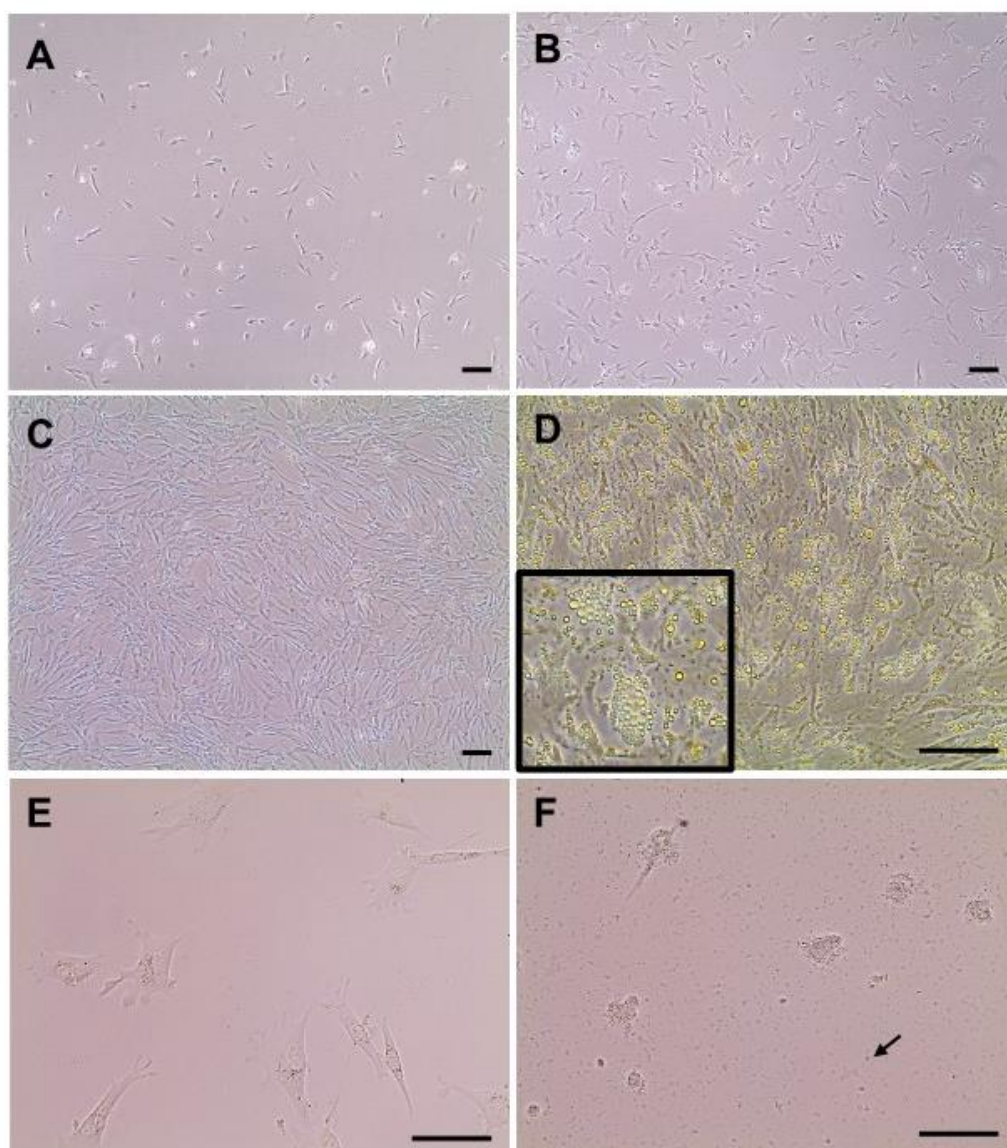


Figure 2.3 Cell morphology comparisons of isolated primary preadipocytes

(A) Preadipocytes at 24 h after isolation. (B) Preadipocytes at 48 h after isolation. (C) Preadipocytes with 80% confluency at 72 h after isolation. (D) Preadipocytes after 48 h adipogenic induction at passage 4, lipid droplets are visible. Inset indicates magnified image of lipid droplets. (E) Representative image of damaged cells. (F) Arrow indicates black swimming dots in contaminated culture. Scale bars = 100 μm .

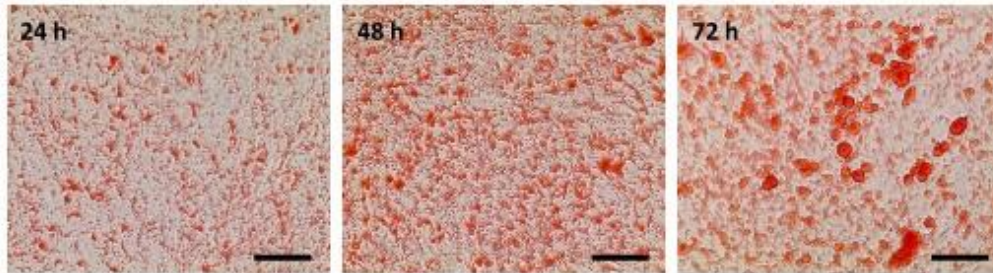
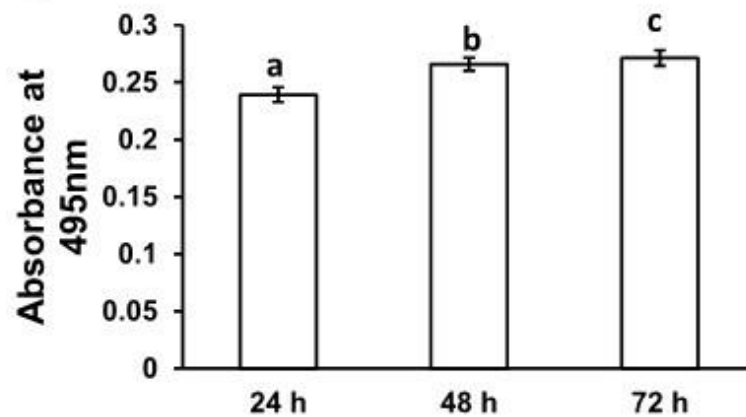
A**B**

Figure 2.4 Evaluation of lipid accumulation by Oil Red O staining

(A) Representative images of Oil Red O staining of E16 preadipocytes after 24 h, 48 h, and 72 h adipogenic differentiation ex vivo. Scale bars = 100 μm . (B) Quantification of lipid droplets measured by elution of Oil red O staining. Values are expressed as mean $\pm$ SD. ^{a,b,c} $P < 0.05$ by one-way ANOVA with Tukey's HSD test.

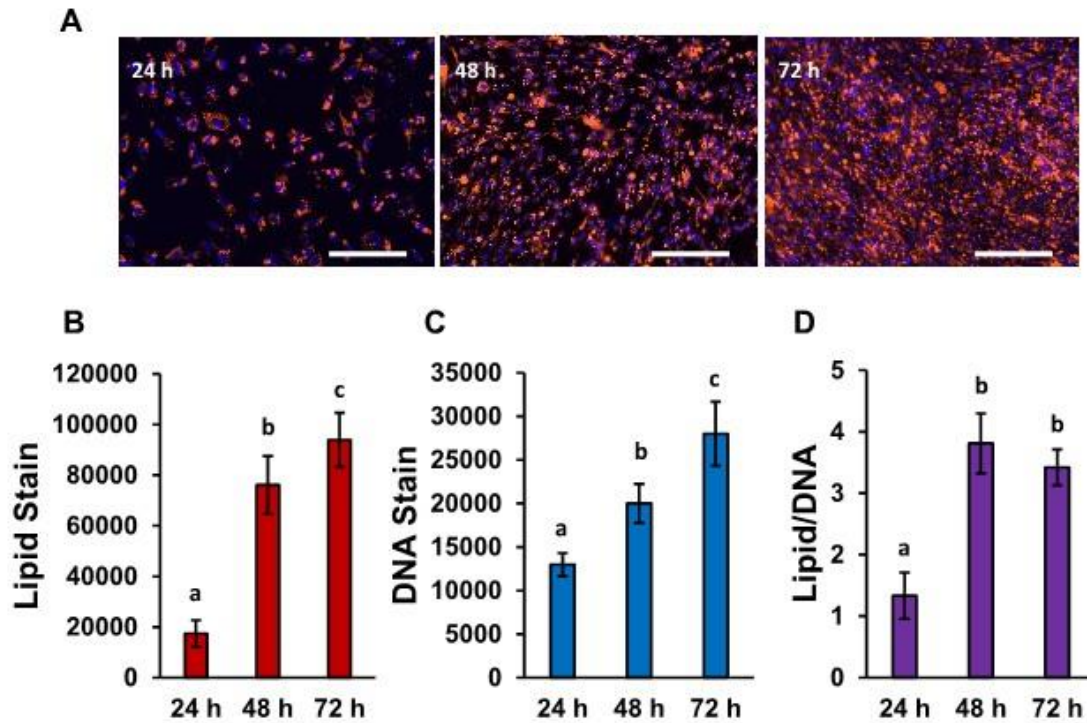


Figure 2.5 Assessment of adipocytes differentiation by lipid stain/DNA stain

(A) Representative images of the lipid stain (red) and the DNA (blue) staining of E16 preadipocytes after 24 h, 48 h, and 72 h adipogenic differentiation ex vivo. Scale bars = 150 μ m.

(B) Lipid staining (Excitation: 485 nm/Emission: 572 nm) was performed to assess lipid accumulation in differentiated preadipocytes. (C) DNA staining (Excitation: 359 nm/Emission: 450 nm) was performed to assess variations in the number of cells. (D) The ratio of lipid and DNA

stain. Lipid accumulation is normalized to the DNA content. Values are expressed as mean $\pm$ SD.

^{a,b,c} $P < 0.05$ by one-way ANOVA with Tukey's HSD test.

CHAPTER 3

EFFECTS OF MATERNAL FISH OIL ON ADIPOSE DEVELOPMENT IN THE BROILER CHICK EMBRYO

A version of this chapter is being prepared for publication in *Poultry Science* by Minjeong Kim and Brynn Voy.

Abstract

Broiler chicks rapidly accumulate adipose tissue after hatch by converting feed to excess fat instead of muscle, which wastes feed, the most expensive component of production. We previously demonstrated that fat accretion in broilers can be reduced by supplementing the broiler hen diet with fish oil, a source of omega-3 polyunsaturated fatty acids (**n-3 PUFA**), eicosapentaenoic acid (**EPA**, 20:5n-3) and docosahexaenoic acid (**DHA**, 22:6n-3). Our objective here was to characterize the effects of maternal dietary fish oil on adipose development in broiler embryos. Broiler-breeder hens were fed diets containing 2.3% of either fish oil or soybean oil (n-6 PUFA control) for four weeks. Fertilized eggs from maternal fish oil (**MFO**) and soybean oil (**MSO**) were randomly collected and incubated to embryonic ages (**E**) 14, E16, and E20. Quantitation of fatty acid profiles revealed that MFO significantly enriched yolk and subcutaneous adipose tissue in EPA and DHA. Histological analysis exhibited that MFO significantly reduced adipocyte size, increasing the frequency of small adipocytes at each age examined. Preadipocytes isolated from E16 adipose tissue in MFO accumulated significantly less lipid than MSO controls, consistent with inhibition of fat accretion by fish oil. In addition, MFO preadipocytes exhibited a significantly higher mitochondrial activity than MSO preadipocytes, attributed to their increased density, indicating improved oxidative metabolism capacity. Using targeted RNAseq, only modest effects of diet on gene expression were observed at E14, when adipose tissue begins to appear in the embryo. By E16 and E20, however, 30 and 60 genes, respectively, were differentially expressed between MFO and MSO (FDR $P < 0.05$), many of which differed at both ages. These sets of genes included key

regulators of adipogenesis, metabolism, and stem cell commitment. Collectively, these results indicate that dietary programming by n-3 PUFA may reduce fat accretion after hatch through effects on adipogenesis that begin in the embryo.

Introduction

Modern broiler chickens, a popular food source for humans worldwide, are incredibly efficient for meat production due to intensive genetic selection for rapid growth and muscle yield over several decades. However, selection has also enhanced accretion of adipose tissues in broilers (Siegel, 2014; Zuidhof et al., 2014; Moreira et al., 2018). As a result, broiler chickens are likely to accumulate excessive fat by utilizing feed that could otherwise be used for primary meat production. This poses a challenge for poultry producers as it leads to depressed feed efficiency, which is the highest component of production cost, and thus reduced profitability. Although subcutaneous and abdominal adipose tissue play crucial roles in energy storage during the embryonic and post-hatching period respectively, fat is not commercially valuable in meat-producing birds and is rather considered one of the main wastes in the production process. Moreover, as the global human population continues to grow, strategies to reduce excess fat accumulation in broiler meat production will be increasingly important to meet the increasing demand for animal proteins.

Broiler chickens now reach market weight much earlier than a few decades ago due to advanced breeding programs that promote rapid growth. This has contributed to an improvement in feed efficiency by slaughtering chickens at an earlier age, and thus relative fat content seemed to be reduced to some extent since body fat relatively increases with aging (Baéza and Le Bihan-Duval, 2013; Zuidhof et al., 2014). Nevertheless, as chickens grow more quickly, they tend to

deposit more body fat because the growth rate is positively correlated with fatness (Leclercq, 1988; Rance et al., 2002; Zerehdaran, 2005; Suzuki et al., 2019). Consequently, the birds still accumulate more fat than is physiologically necessary due to rapid growth. Given the high heritability of fatness in broiler chickens, selecting against fatness seemed to be a promising approach to reducing fat deposition in broiler chickens. However, selecting chicken lines that exhibit both increased muscle weight and reduced fat deposition is complex and challenging because the growth rate has a positive correlation with body weight, in addition, body weight is positively correlated with fat content as well as muscle weight (Leclercq et al., 1980; Le Bihan-Duval et al., 1998; Chabault et al., 2012; Demeure et al., 2013). Indeed, the results are inconsistent between studies performed with selected broilers based on fat content and vary greatly depending on age and sex (Ricard et al., 1983; CAHANER and NITSAN, 1985; Leclercq, 1988; Gaya et al., 2006; Wu et al., 2006; Wang et al., 2021). As breeding and selection are processes that require a long-term commitment, more effective strategies for reducing fatness in broiler chickens while maintaining their growth potential are still needed.

Unlimited access to feed is another factor contributing to excessive body fat content in commercial broilers. Excessive energy intake beyond metabolic needs results in the accumulation of excess energy in the form of triglycerides in adipose tissue, leading to undesirable fat accretion in broilers. Therefore, restricted feeding regimens have been considered to control growth rate and fatness. Previous studies have proposed the possibility of feed restrictions to produce broilers with reduced body fat, without affecting body weight through a negative energy balance (Jones and Farrell, 1992; Santoso et al., 1993; Sahraei, 2012). Effective early intervention is crucial in combating fatness in broiler chickens since fat accretion begins shortly after hatch. The first two

weeks of a chicken's life are a critical period for fat development, which can affect its fatness later in life (Hermier et al., 1989).

For example, restricting feed intake during the early stages of growth in chickens led to reduced carcass fat contents and abdominal fat deposition, along with improved feed efficiency (Plavnik et al., 1986). However, there are also studies that show contradictory results. Early feed restrictions in broiler chickens have not been shown to reduce fatness when the birds reach market age (42 – 56 d of age) (Santoso et al., 1995; Zubair and Leeson, 1996; Butzen et al., 2015). Inadequate nutrient supply during the early stages of life can have long-lasting negative effects on the growth, tissue development, and overall health of young chicks, leading to welfare concerns such as hunger and stress (Mench, 2002; De Jong and Guemene, 2011; Morrissey et al., 2014; Van Krimpen and De Jong, 2014; Yan et al., 2021). Zhan et al. (2007) reported that broilers subjected to early feed restriction had higher abdominal fat content at the end of the study compared to birds that were not feed-restricted. As such, early feed restriction may disrupt the normal metabolic and physiological development of broiler chickens, leading to adverse effects on body composition. Furthermore, feed restriction can cause decreased muscle weight or require an extended feeding period to achieve the target slaughter weight, despite previous beliefs that such restrictions would lead to leaner meat (Lippens et al., 2000; Mohebodini et al., 2009; Van der Klein et al., 2017; Tůmová et al., 2021; Yan et al., 2021). Given that the effectiveness and persistence of feed restriction in reducing fat in broilers greatly depend on factors such as the duration and intensity of the restriction, there may be a need to develop more sophisticated and systematic guidelines for feed restriction to effectively address the issue of fatness.

Dietary nutrients, particularly dietary fat sources, have a significant impact on the development and growth of adipose tissue. Omega-3 polyunsaturated fatty acids (**n-3 PUFA**) have

been shown to have anti-obesogenic effects in vitro, and in a number of species in vivo. This class of fatty acids can attenuate lipid accumulation by regulating genes associated with adipogenesis and promoting fatty acid oxidation, lipolysis, and adipose thermogenesis (Crespo and Esteve-Garcia, 2002b; c; Azain, 2004; Todorčević and Hodson, 2015; Chen et al., 2020; Zapata et al., 2022; Raj et al., 2023). Eicosapentaenoic acid (**EPA**; C20:5n-3) and docosahexaenoic (**DHA**; C22:6n-3) acids are the main contributors to the anti-obesogenic effects of n-3 PUFA. Long-chain n-3 PUFA cannot be synthesized in most vertebrates; rather, they are acquired from the diet. In contrast cold-water marine algae are highly efficient at synthesizing long-chain n-3 PUFA, and are rich sources of EPA and DHA. Dietary sources of EPA and DHA for humans are primarily derived from cold-water marine fish species that consume these algae. Fish oil is therefore a common source of EPA and DHA for both humans and for experimental studies in animals. Previous studies have demonstrated that supplementing feed with fish oil, either alone or in combination with other n-3 PUFA sources, can improve body composition by inhibiting adipose tissue development and reducing fat accumulation in chickens (Gonzalez-Ortiz et al., 2013; Torchon et al., 2017; Ibrahim et al., 2018; Kim and Voy, 2021). A recent study reported that enriching the broiler diet with fish oil (at 0.05%, 0.1%, and 0.15%) for 3 weeks increased breast muscle weight and decreased abdominal fat content when compared to the control group (Wahid et al., 2023). The study also indicated that the impact of dietary fish oil on broiler body composition was more pronounced with higher doses of fish oil added to the diet. Additionally, the supplementation of fish oil did not have any negative effects on the quality of the meat produced, such as meat color and off-flavor. Collectively, these studies support the concept that adipose deposition in broilers can be regulated by fish oil in the diet. However, the cost of supplementing fish oil in poultry feed can be a limiting factor for large-scale commercial broiler production, especially considering the long feeding

duration until market age. Although some consumers are willing to pay the extra cost of these products to some extent, the increasing interest in animal welfare and preference for healthy products among consumers in recent years emphasizes the importance of developing practical and cost-effective strategies for effectively utilizing fish oil in commercial broilers.

Enriching hens' diet with fish oil, which is rich in n-3 PUFA, can be a practical way to utilize its benefits in commercial broiler production. The type of fatty acids consumed by the mother during pregnancy can have a significant impact on fetal fat development and deposition. Studies from humans and mice indicated that n-3 PUFA in the maternal diet can program the susceptibility and resistance of offspring to obesity (Vidakovic et al., 2016a; Rudolph et al., 2018; Saros et al., 2023). In the poultry industry, this relationship between maternal dietary fatty acids and the adiposity of offspring is particularly significant since the development of chick embryos is greatly influenced by yolk nutrients, which reflect the fatty acid composition of the hen diet. Supplementing hen diets with fish oil has been shown to enrich the yolk with n-3 PUFA, specifically EPA and DHA (Kralik et al., 2021; Ghobashy et al., 2023). These fatty acids, in turn, may play a critical role in supporting tissue growth during embryonic development. Therefore, through the strategic use of fish oil supplementation, the fatty acid composition and nutritional quality for chick development can be improved. Furthermore, enriching the yolk with n-3 PUFA through the hen diet can lead to significant cost reduction compared to feeding fish oil to young chicks and mature birds. This cost-efficient approach allows for the utilization of the anti-obesogenic effects of n-3 PUFA in a more economical manner. In a prior study by Beckford et al. (2017), chicks hatched from hens fed a fish oil-enriched diet showed lower body fat percentage and smaller adipocytes at 7 and 14 days compared to those hatched from hens fed a corn oil-based diet. The study further revealed that the fish oil-enriched diet of hen affected markers associated

with adipogenesis, fatty acid transport, and lipid metabolism in the adipose tissue of post-hatch chicks. These findings suggest that enriching the hen diet with fish oil has the potential to effectively program reduced fat accumulation in post-hatch chicks by modulating adipogenesis and lipid metabolism during the embryonic development stage. However, there is still a knowledge gap regarding the impact of these fatty acids present in the yolk on adipose development, metabolism, and body composition in chick embryos. Filling this gap is crucial for developing practical and effective ways to limit adiposity and promote lean growth in broiler chickens. Therefore, this study aimed to investigate the effects of maternal fish oil supplementation on adipose tissue development in broiler chick embryos, with the objective of establishing a foundation for future studies focused on reducing fat accumulation in post-hatch broilers.

Materials and methods

Experimental Animals

Ethics Statement. All animal procedures involving broiler breeder hens were approved by Institutional Animal Care and Use Committee of the University of Georgia. Embryos were euthanized following guidelines provided by the Institutional Animal Care and Use Committee of the University of Tennessee.

Husbandry and Diet. Ross 708 broiler breeder hens were raised at the Poultry Research Farm of University of Georgia (Athens, GA). Hens (n=30/diet) were fed standard broiler-breeder diets containing 2.3 % (wt:wt) of either soybean oil (**SO**; Conagra Brands, Chicago, IL) or fish oil (**FO**; Jedwards International, Braintree, MA) ad libitum for 4 weeks to fully enrich eggs with the corresponding fatty acids. Formulations for each diet are shown in Table 3.1 and the fatty acid

composition of each diet is shown in Table 3.2. The final n-3 content of the two diets was 2.2% (SO) and 5.2% (FO).

Egg Incubation. Freshly fertilized broiler eggs (n=36/diet) were obtained from the maternal fish oil (**MFO**) group or soybean oil group (**MSO**) and transferred to the Johnson Animal Research and Teaching Unit of University of Tennessee (Knoxville, TN). Eggs were labeled and placed in a digital incubator (Ova-Easy 580 Advance Series II cabinet incubator, Brinsea, FL, USA) at 37.5°C with 55-60% relative humidity, with automatic egg turning around the long axis (45°) every 30 min. Incubation temperature and humidity were monitored twice a day until dissection. Fertilized eggs were incubated to embryonic ages (**E**) 14, 16, and 20 spanning the phase of intense embryonic growth.

Tissue Collection

Embryos from each maternal dietary group were euthanized at E14, E16, and E20 (n=12/diet/ age) by cervical dislocation and dissected to collect adipose tissue. Weights of the egg, embryo, yolk sac, fat pad (parafemoral) and breast muscle were measured at each age. Relative weights of fat pad and breast muscle were calculated by dividing the tissue weight by the embryo weight. The left fat pad was fixed overnight in 4% paraformaldehyde at 4°C for histology, and the right was snap-frozen in liquid nitrogen, then pulverized under liquid nitrogen to produce a homogeneous powder for both fatty acid analysis and RNA isolation. Yolk sac was also snap-frozen and pulverized for subsequent fatty acid profiling. Samples were stored at -80°C until analysis. At E16, adipose tissue (n=6/diet) was also used to isolate preadipocytes for primary culture and assay of adipogenic potential ex vivo.

Fatty Acid Analysis

Fatty acid profiles of the yolk (E0 and E16) and adipose tissue (E16) were analyzed using gas chromatography-mass spectrometry (**GC-MS**), as previously described (Powers and Campagna, 2019). Approximately 30 mg of pulverized frozen tissue (n=6/diet) was used for extraction of fatty acids and derivatization to their methyl esters. Analyses were performed using a Trace 1300 gas chromatograph (Thermo Fisher Scientific, Waltham, MA) coupled to an Exactive™ Plus Orbitrap mass spectrometer (Thermo Fisher Scientific, Waltham, MA) and using a custom interface equipped with an atmospheric pressure chemical ionization (**APCI**) source. Fatty acid methyl esters (**FAMES**) were identified based on their retention time using a standard reference (Supelco 37 Component FAME mix, CRM47885, Burlington, MA), as previously described (Powers and Campagna, 2019). The fatty acid content of the yolk and adipose tissue was expressed as relative percentages of the total FAMES detected in each sample. Indices of enzyme activities for stearoyl-CoA desaturase (**SCD**), delta-5 desaturase (**D5D**), delta-6 desaturase (**D6D**), *de novo* lipogenesis (**DNL**), and elongation (**EL**) in adipose tissue were estimated by calculating the product to precursor ratios using the following equations: SCD16, 16:1n-7/16:0; SCD18, 18:1n-9/18:0; D5D, 20:4n-6/20:3n-6; D6D, 18:3n-6/18:2n-6; DNL, 16:0/18:2n-6; EL, 18:0/16:0.

Histological Analysis of Adipocyte Size

Fixed adipose depots were embedded, sectioned into 5 µm slices, and subsequently stained with hematoxylin and eosin (**H&E**) using standard methods. Adipose sections stained with H&E were imaged at 40X magnification using an EVOS XL Core Imaging System (AMEX1000, Thermo Fisher Scientific, Waltham, MA). Adipocyte size analysis was performed using a combination method of AdipoCount (version 1.2) and ImageJ (version 1.53k, NIH) software, following the

previously described method with some modifications (Hu et al., 2021). Measures were taken from five embryos/diet/age, from two independent slides/embryo, and in three fields/slide, for a total of 30 fields for each group. At least 200 cells were evaluated per slide.

Adipogenic Potential of Preadipocyte

Isolation of Preadipocytes. Primary preadipocytes were isolated from E16 embryos (n=6/diet) and cultured using a previously optimized method as described by Kim et al. (2022). Adipose tissues obtained from two embryos were pooled together to generate a total of three biological replicates per diet.

Adipogenic Differentiation. Preadipocytes were plated in 96-well plates with 12 wells/biological replicate/diet. Growth media was replaced with adipogenic differentiation media (DMEM/F12 media containing 10% chicken serum (Gibco, Carlsbad, CA), 1X Linoleic Acid-Oleic Acid-Albumin (L9655, Sigma-Aldrich, St. Louis, MO), and 1% penicillin-streptomycin) when cells reached 80% confluence. The degree of differentiation was assessed after 72 h by staining cells with two different lipid-sensitive dyes, Oil Red O (Sigma-Aldrich, St. Louis, MO) and AdipoRed (PT-7009, Lonza, Walkersville, MD), as previously described (Kim et al., 2022). Staining was performed with three technical replicates in each biological replicate. For Oil Red O, lipid accumulation in each well was quantified by extracting the stain in 100% isopropanol and reading the absorbance at 495 nm using a microplate reader (Synergy H1, BioTek, Winooski, VT). AdipoRed staining was normalized to DNA content, determined by staining nuclei with NucBlue reagent (Hoechst 33342; R37605, Thermo Fisher Scientific, Waltham, MA). Quantitation of AdipoRed and NucBlue was performed on a fluorescent plate reader (Synergy H1, BioTek, Winooski, VT) by detecting the lipid stain with excitation of 485 nm and emission of 572 nm, and

the DNA stain with excitation of 359 nm and emission of 450 nm. Images of the AdipoRed and NucBlue stained cells were captured using a fluorescent microscope (EVOS M7000 imaging system, Thermo Fisher Scientific) at 40X magnification for the purpose of visualization.

Mitochondrial Analysis. Mitochondrial activity was confirmed by measuring mitochondrial membrane potential (**MMP**), which specifically reflects mitochondrial respiration, by staining cells with the membrane potential-dependent probe MitoTracker Red CM-H2XRos (M7513, Invitrogen, Carlsbad, CA). Additionally, mitochondrial mass was detected using MitoTracker Green FM (M7514, Invitrogen, Carlsbad, CA). Staining procedures were conducted as specified by manufacturer's instructions. Briefly, the culture media was removed from the dish, and a prewarmed staining solution containing MitoTracker probes (500 nM) was added, followed by incubation under growth conditions. After 45 min incubation, the staining solution was replaced with serum-free media. Accumulation of fluorescent probes within the mitochondria was then observed using a fluorescent microscope (EVOS M7000 imaging system, Thermo Fisher Scientific) equipped with Texas Red (red) and GFP (green) filters. Images were captured at 40X magnification, and the integrated density of the acquired images was determined using the ROI manager in the ImageJ program. Mitochondrial activity and content were quantified based on the fluorescence intensity of red and green signals, respectively. The relative mitochondrial activity was then normalized to the mitochondria content.

Gene Expression Analysis by Targeted RNA sequencing

RNA Isolation and cDNA Synthesis. Approximately 25 mg of pulverized adipose tissue (n=12/age/diet) was used to isolate total RNA, using TRIzol reagent (15596026, Invitrogen, Carlsbad, CA) and Direct-Zol RNA Miniprep kit (R2050, Zymo Research, Irvine, CA) according

to the manufacturer's instructions. RNA concentration was measured using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA), and the purity was assessed based on A260/A280 ratio. The integrity of isolated RNA was visualized through MOPS gel electrophoresis. After verifying RNA quality and integrity, cDNA was synthesized from 500 ng of total RNA using the iScript cDNA synthesis kit (Bio-Rad, Hercules, CA).

Targeted RNA sequencing. Expression of a panel of 504 genes related to adipose development, differentiation, and metabolism was analyzed using targeted RNA sequencing, as previously described (Mihelic et al., 2020; Jung et al., 2022). Barcoded amplicons were sequenced on a NovaSeq X Plus device running a 2 × 150 bp paired-end configuration (Illumina, San Diego, CA). Raw sequence FASTQ files were mapped and annotated to the chicken (*Gallus gallus*) reference genome (bGalGal1.mat.broiler.GRCg7b) using QIAGEN CLC Genomics Workbench 23 with default parameters. Differentially expressed genes (**DEGs**) between MSO and MFO groups were identified using the edgeR package v 3.17 in R 4.3.1 (Chen et al., 2020b) after filtering for low expression, requiring at least 50 read counts in all samples. The trimmed mean of M values (**TMM**) normalization was applied to estimate effective library sizes, and multiple testing correction was performed using the False Discovery Rate (**FDR**) method. Venn diagram was created to visualize the number of DEGs (FDR-adjusted $P < 0.05$) that are regulated across developmental age and by hen diet. Differentially expressed genes were visualized using MetaboAnalyst 5.0 online platform and the Database for Annotation, Visualization, and Integrated Discovery (DAVID, v2023q2) was used for Kyoto Encyclopedia of Genes and Genomes (**KEGG**) analysis.

Statistical Analysis

Data other than those from targeted RNAseq were analyzed using JMP Pro (version 17, SAS Institute Inc., Cary, NC), and the values were expressed as the mean $\pm$ SEM. Significant differences between the group means were determined by Student's t-test or one-way ANOVA using Tukey–Kramer HSD test at $P < 0.05$, unless otherwise specified.

Results

Embryonic Growth Performance

Effects of MSO and MFO on embryo growth and development of adipose and breast muscle were measured over three embryonic ages, E14, E16, and E20. Egg weight, embryo weight, and residual yolk sac weight did not differ significantly between MSO and MFO at any age (data not shown). In addition, there were no significant effects of hen diet on leg fat pad weight or breast muscle weight (absolute or relative) at any time point.

Fatty Acids Composition of Yolk and Adipose Tissue

Fatty acid compositions of the yolk and adipose tissue were measured to quantify the level of enrichment due to hen diet and to monitor the uptake and mobilization in developing adipose tissue. As expected, MFO significantly increased levels of eicosapentaenoic (EPA; C20:5n-3) and docosahexaenoic (DHA; C22:6n-3) acids in the initial yolk (E0), and these differences persisted in the residual yolks at E16 (Figure 1). Conversely, levels of arachidonic acid (AA; C20:4n-6) were significantly higher in MSO vs. MFO of both E0 and E16 yolk. Total levels of saturated (SFA), monounsaturated (MUFA), and polyunsaturated (PUFA) fatty acids did not differ significantly in yolk between the two diet groups at E0. Overall fatty acid content in the initial

(E0) and E16 residual yolk is shown in Table 3.3. Relative abundances of myristic acid (C14:0), myristoleic (C14:1n-5), palmitoleic (C16:1n-7), erucic (C22:1n-9), and nervonic (C24:1n-9) acids were higher in MFO vs. MSO at both E0 and E16 ($P < 0.05$). Content of palmitic acid (C16:0) was higher, and stearic acid (C18:0) level was lower in MFO vs. MSO at E16 ($P < 0.05$) but did not differ significantly at E0. As described in Figure 3.1, the higher levels of EPA and DHA in MFO yolks resulted in an increased total content of n-3 PUFA compared to yolks from MSO ($P < 0.05$). Likewise, the total content of n-6 PUFA was significantly higher in MSO yolks with the increases in linoleic (LA; C18:2n-6) and AA (C20:4n-6). The contents of γ -linolenic (GLA; C18:3n-6), eicosadienoic (C20: 2n-6), and dihomo- γ -linolenic (DGLA; C20:3n-6) acids were also higher in MSO at E16 ($P < 0.05$). Therefore, the n-6/n-3 ratio was significantly higher in MSO at E16 compared to MFO. No effect of maternal diet on total SFA and MUFA was observed.

Differential enrichment of the yolk in n-3 and n-6 PUFA was reflected in embryonic adipose tissue, which develops by accumulating fatty acids mobilized from the yolk (Table 4). At E16, relative levels of EPA and DHA were approximately 9- and 4.5-fold greater in adipose of MFO vs. MSO ($P < 0.001$), respectively. Conversely, levels of LA (C18:2 n-6) and all of its downstream products (C18:3 n-6, C20:2n-6, C20:3n-6, and C20:4n-6) that were detected in our analysis were significantly increased in adipose of MSO vs. MFO. Three long chain fatty acids of very low relative abundance (C13:0, C14:0, C15:0) also differed significantly between MSO and MFO, presumably due to modest differences between soybean and fish oils. Total relative levels of SFA, MUFA, and PUFA did not differ in E16 adipose of the two groups. In addition to effects that would be predicted based on the differential content of LC n-3 and n-6 PUFA species in the hen diets, MFO also significantly increased abundance of palmitoleic acid (C16:1n-7) in adipose tissue (1.7 vs. 2.1%, $P = 0.008$). The activities of fatty acid desaturases and indices of adipose

tissue *de novo* lipogenesis (DNL) and elongation (EL) were estimated from the product-precursor ratios. As shown in Table 3.4, MFO showed evidence of a higher stearyl-CoA desaturase-16 (SCD16, $P = 0.042$) activity, while delta-5 desaturase (D5D) was significantly higher in MSO. Indices of SCD18 and D6D were not different between MSO and MFO. In addition, DNL index was higher in MFO ($P = 0.002$), whereas the level of EL was higher in MSO ($P = 0.047$).

Effect of MFO on Adipose Growth

The morphological characteristics of subcutaneous adipose tissue were histologically evaluated at E14, E16, and E20 (Figure 3.2A). Numerous small, unilocular adipocytes and clusters of nuclei were visible in histological images of E14. Expansion of adipocytes was noticeable in the images of older embryonic ages. Consistent with the histological images, the average adipocyte size increased during development (Figure 3.2B). On average, adipocytes were consistently and significantly larger in MSO than MFO at each age group (E14, 254 ± 18 vs. $174 \pm 9 \mu\text{m}^2$; E16, 415 ± 11 vs. $336 \pm 21 \mu\text{m}^2$; E20, 697 ± 7 vs. $573 \pm 13 \mu\text{m}^2$, MSO vs. MFO, respectively). These differences resulted from a shift in the relative distribution of adipocytes by size, with MFO adipose exhibiting a higher frequency of smaller adipocytes and a reduced frequency of larger adipocytes (Figure 3.3). Differences in adipocyte distribution were apparent as early as E14, at which the proportion of small adipocytes $< 100 \mu\text{m}^2$ was higher in MFO (15% vs. 31%, $P < 0.01$) than in MSO, with a significantly higher proportion of large adipocytes ($200\text{-}600 \mu\text{m}^2$, 58% vs. 36%) in MSO. Differences persisted as adipose development proceeded, even as adipocyte size increased in both diet groups. At E16, the proportion of MFO adipocytes in the smaller range ($< 200 \mu\text{m}^2$) was significantly higher than in MSO, while MSO exhibited a greater proportion of large adipocytes ($400\text{-}800 \mu\text{m}^2$, $P < 0.01$). This pattern was continued during adipose development at

E20. These data illustrated that the types of fatty acids in the hen diet influenced adipose development, especially adipocyte size in the embryo.

Effect of MFO on Adipogenic Progenitor Cells

Reduced adipocyte size in MFO vs. MSO embryos suggested that the hen diet differentially influenced lipid accumulation in developing adipocytes. To test this possibility in vitro, primary preadipocytes were isolated from MFO and MSO embryos at E16 and induced to undergo adipogenic differentiation in vitro (Figure 3.4). After 72 h of adipogenic induction, developing adipocytes from MFO accumulated significantly less lipid than MSO, based on Oil Red O staining (Figure 4B). Independent replicates of cells were dually-stained for lipid content and DNA, to more specifically assess lipid accumulation on a per cell basis and to confirm that differences observed with Oil Red O staining were not due to differences in cell number. Consistent with the results from Oil Red O, MFO preadipocytes induced to undergo adipogenic differentiation in vitro accumulated less lipid on both a total and per cell basis than those isolated from MSO (Figure 4C-E). These differences were not due to a reduction in viability of MFO preadipocytes, as NucBlue staining did not differ between diet groups. Mitochondrial activity was assessed to determine if effects of MFO on metabolic rate could contribute to differences in lipid accumulation (Figure 3.5). Compared to MSO, MFO preadipocytes exhibited significantly higher mitochondrial activity due to higher mitochondrial content ($P < 0.001$), indicating an enhanced capacity for oxidative metabolism.

Effect of MFO on Molecular Profiling of Adipose Tissue

Adipose tissue develops rapidly over the 6 days (E14-E20) profiled herein. These changes were reflected in differential gene expression, with 386 genes differing significantly between at

least one pairwise comparison based on age alone. The expression of 147 genes significantly differed in all age comparisons (Figure 3.6 and Supplementary Table 1). KEGG pathway enrichment analysis revealed that these overlapping DEGs were primarily enriched in pathways associated with adipogenesis and lipid accumulation, including biosynthesis of unsaturated fatty acids, fatty acid metabolism, c-type lectin receptor signaling pathway, apelin signaling pathway, adipocytokine signaling pathway, Wnt signaling pathway, PPAR signaling pathway, FOXO signaling pathway, focal adhesion, and MAPK signaling pathway. To investigate changes in key adipogenic gene expression during the development of chick embryo adipose tissue, we examined the expression of markers representing adipocyte progenitors (CD24, CD36), differentiating preadipocytes (bone morphogenetic protein 2, BMP2; CCAAT enhancer binding protein beta, CEBP β ; CCAAT enhancer binding protein alpha, CEBP α ; sterol regulatory element binding transcription factor 1, SREBF1; peroxisome proliferator-activated receptor gamma, PPAR γ), mature adipocytes (fatty acid binding protein 4, FABP4; adiponectin, C1Q and collagen domain-containing, ADIPOQ; lipoprotein lipase, LPL; patatin-like phospholipase domain-containing 2, PNPLA2, also known as adipose triglyceride lipase, ATGL; fatty acid synthase, FASN), insulin signaling (insulin receptor substrate 1, IRS1; AKT serine/threonine kinase 1, AKT1), and adipocyte inflammation (interleukin 6, IL6) across different embryonic ages. As shown in Figure 3.7, adipogenic markers were significantly different at E16 and E20 compared to E14 (FDR $P < 0.05$). E14 to E16 represents a period of marked changes in adipose development at the molecular level, based on changes in expression of this set of genes. The majority of changes in expression at E16 persisted, or were enhanced further, at E20. Expression of adipocyte progenitor markers CD24 and CD36 was up-regulated during adipose development. In comparison to E14, the expression of adipogenesis markers BMP2, CEBP β , and PPAR γ was significantly higher, while

the expression of CEBP α and SREBF1 was lower in both E16 and E20 adipose tissues. Regarding mature adipocyte markers, the expression of FABP4, ADIPOQ, LPL, and PNPLA2 was significantly higher, whereas FASN showed lower expression in E16 and E20 adipose tissue when compared to E14. The expression of markers related to insulin signaling (IRS1, AKT1) and adipocyte inflammation (IL6) also increased as adipose expansion progressed.

Of the 384 genes that were differentially expressed across development, 76 were also significantly affected by the hen diet in at least one of the three embryonic ages (Figure 3.8 and Supplementary Table 2). KEGG pathway analysis showed that these overlapping genes were mainly enriched in biosynthesis of steroid and unsaturated fatty acids, fatty acid metabolism, c-type lectin receptor signaling pathway, apelin signaling pathway, cytokine-cytokine receptor interaction, and MAPK signaling pathway (Figure 3.8C). Pairwise comparisons between MFO and MSO revealed a progressive effect of hen diet on gene expression across the three ages studied. At E14, only 1 gene related to adipogenesis and lipid metabolism, LDL receptor related protein 1 (LRP1) differed significantly between MFO and MSO (FDR $P < 0.05$), when adipose tissue begins to appear in the embryo (Supplementary Table 3). By E16, this number of genes increased to 30, of which 11 genes were expressed at higher levels and 19 genes at lower levels in MFO vs. MSO (Supplementary Table 4). These sets of genes are mainly associated with embryonic organ development, cell fate commitment, lipid localization, fatty acid transport, and mitochondrial organization. Finally, at E20, expression of a total of 60 genes differed significantly between MFO and MSO, including with higher levels and 21 with lower levels of expression (Supplementary Table 5). These DEGs are not only involved in functions related to developmental growth, cell differentiation, and lipid biosynthetic processes but are also associated with glucose metabolism and thermogenesis. Over three embryonic ages, there were 11 common DEGs between MFO and

MSO, identified at both E16 and E20. A set of 10 genes showed consistent expression patterns at both embryonic ages, with 2 up-regulated and 8 down-regulated genes in MFO vs. MSO (Figure 3.9). Among these, transcription elongation regulator 1 (TCERG1) and peptidase M20 domain containing 1 (PM20D1) were significantly up-regulated by MFO at both ages. Genes expressed at lower levels in MFO included tubulin beta 4B class IVb (TUBB4B), angiotensin I converting enzyme (ACE), Cbp/p300 interacting transactivator with Glu/Asp rich carboxy-terminal domain 4 (CITED4), insulin like growth factor 1 receptor (IGF1R), solute carrier family 25 member 28 (SLC25A28), PR/SET domain 16 (PRDM16), bone morphogenetic protein 2 (BMP2), and CD24 molecule (CD24). The expression of DNA topoisomerase II alpha (TOP2A) was lower in MFO at E16 but higher at E20. These common DEGs are involved in transcription regulation (TCERG1, PRDM16, and BMP2), cell proliferation (TOP2A, TUBB4B, and ACE), adipogenic differentiation (CITED4, IGF1R, SLC25A28, PRDM16, BMP2, and CD24), thermogenesis and energy expenditure (PM20D1, ACE, and PRDM16), and obesity (TUBB4B and BMP2).

Discussion

This study builds upon previous work in our lab, in which we demonstrated that enriching the hen diet in fish oil significantly reduced adiposity in broiler chicks after hatch. The objective here was to determine if these differences originate during embryonic development of adipose tissue and to identify underlying molecular effects of including fish oil in the hen diet. The concept of programming adipose development in broiler chicks through fats in the hen diet is largely unexplored. However, it has the potential to be utilized in broiler production because of the ease with which the yolk fatty acid profile can be manipulated through the hen diet. Understanding the mechanisms through which fatty acids alter aspects of development that are relevant to body

composition is therefore potentially valuable for the broiler industry. In addition, this model can yield valuable insight that is relevant to childhood obesity. Several studies have associated n-3 PUFA in the maternal diet with reduced adiposity in children, but the ability to conduct mechanistic studies into this relationship is limited in humans. In contrast, the source of fatty acids from which the embryo develops can be precisely modelled in avians due to the role of the yolk and to development in ovo. This study is therefore driven by the gap in knowledge about how different types of fatty acids shape embryonic adipose development and ultimately adiposity, metabolism, and body composition.

Adipocyte size is both a metric that reflects fat accretion and energy storage and a physiological variable that influences metabolic health and energy utilization. We found that adipocytes from MFO were significantly smaller, based on cross-sectional area, than those of MSO at each age examined. Differences in average area resulted from a shift towards a higher frequency of smaller adipocytes and a lower frequency of larger ones in MFO vs. MSO. These effects of the hen diet are consistent with our previous work, in which we found a significant increase in prevalence of small adipocytes and decrease in frequency of large adipocytes in MFO vs. MSO at 7 and 14 d after hatch, using the same hen feeding protocol used herein (Beckford et al., 2017). They are also consistent with feeding studies conducted after hatch that have shown reduced adipocyte size when broilers consume diets enriched in fish oil (Gonzalez-Ortiz et al., 2013; Torchon et al., 2017). Adipocyte development in the chick embryo becomes visible around E12 with accumulation of lipids in multiple cellular droplets. As cells begin to mature, droplets fuse to form unilocular cells and the droplet expands as fatty acids are mobilized from the yolk, assembled into triglycerides, and stored in the adipocyte. A delay in this step of adipogenesis has been suggested to underlie the smaller adipocyte size observed in broiler chick embryos compared to

those of Leghorns (Chen et al., 2014). Therefore, slower rates of this early step of adipocyte maturation could have contributed to reduced adipocyte size observed in MFO vs MSO. Although the study described herein focused on effects of MFO in the embryo, we previously reported that the differences in adipocyte size between MFO and MSO were maintained to at least 14 d of age (Beckford et al., 2017). This shift to favor the abundance of smaller adipocytes may support rapid growth by facilitating the mobilization of stored fatty acids for use by muscle and other intensively growing tissues. Limiting the expansion of adipocyte lipid droplets enables more efficient lipolysis due to increased droplet surface area (Qian et al., 2023). Of note, we previously demonstrated that MFO broilers raised to market age exhibited an increase in lean mass relative to MSO, although we did not assess lipolytic activity as a basis for that growth. This early effect of the maternal diet on adipocyte size also has implications for childhood obesity. Hypertrophic adipocytes are associated with metabolic disorders such as obesity and diabetes, primarily due to increased systemic insulin resistance (Ghaben and Scherer, 2019). Moreover, adipocyte hypertrophy can result in tissue hypoxia that in turn contributes to adipose tissue inflammation and secretion of pro-inflammatory cytokines (Skurk et al., 2007; Halberg et al., 2009; Meyer et al., 2013). In contrast, small adipocytes are associated with reduced susceptibility to metabolic disorders (Lönn et al., 2010; Yang et al., 2012).

The effects of MFO on lipid accumulation during adipogenesis were preserved when E16 primary preadipocytes were induced to undergo adipogenic differentiation *ex vivo*. Primary cells from both MSO and MFO were propagated under standard culture conditions, with no media supplementation to maintain differences in fatty acid availability. Under these conditions, MFO accumulated significantly less triglyceride, which suggests that the effects of the hen diet are incorporated into preadipocytes, potentially through persistent effects on the transcriptome.

Previous *in vitro* studies have reported that supplementation of culture media *in vitro* with EPA and DHA reduced lipid accumulation and adipogenesis in adipocytes from various species (Manickam et al., 2010; Barber et al., 2013; Murali et al., 2014; Ferguson et al., 2019). Our model differs from these studies in that media did not include added fatty acids from fish oil; rather, preadipocytes were exposed to DHA and EPA during their *in ovo* development but were tested for adipogenic potential in response to supplementation of media with oleic and linoleic acids (a standard differentiation protocol for chicken preadipocytes). Mechanistically, reduced lipid accumulation in MFO vs. MSO adipocytes *ex vivo* may have resulted from properties conferred by enrichment of cell membrane phospholipids in EPA and DHA, although we did not analyze this effect of the hen diet. Alternatively, reduced lipid accumulation could have resulted from epigenetic modulation of genes that regulate adipogenesis, which has been demonstrated in other models (Khaire et al., 2015).

Our working hypothesis is that fish oil, when added to the hen diet, reduces adiposity through the effects of its characteristic LC n-3 PUFA species. As expected, these fatty acids were significantly more abundant in the yolk and in developing adipose tissue of MFO vs. MSO embryos. However, it is worth noting that another fatty acid, palmitoleic acid (16:1 n-7) may have contributed the effects on adipose development. Levels of palmitoleic acid were significantly enriched in both the yolk and adipose tissue of MFO. This fatty acid, a desaturase product of palmitate (16:0), has been described as a lipokine that enhances energy expenditure in adipocytes and other cell types oxidation (Bolsoni-Lopes et al., 2013; Bolsoni-Lopes et al., 2014; Cruz et al., 2018). Differentiation of 3T3-L1 preadipocytes in the presence of 16:1 n-7 has been shown to enhance energy expenditure and fatty acid utilization compared to adipocytes that formed in the presence of 16:0 (Cruz et al., 2018).

The embryonic ages at which we profiled the effects of dietary lipids represent distinct stages of adipose development. From E12, when adipocytes begin to appear, through E18, hypertrophy proceeds in a linear manner in relation to uptake and storage of lipids from the yolk. After this point, adipocytes become sensitive to lipolytic stimulation and mobilization of lipid for energy begins to occur in preparation for hatch. Only one gene, LRP1, differed significantly in expression level between MSO and MFO at E14. A study by Masson et al. (2009) demonstrated that LRP1 is highly expressed in preadipocytes during adipogenesis in both mice and humans. Silencing LRP1 expression with siRNA1 significantly inhibited the mRNA levels of key adipocyte differentiation markers (PPAR γ , HSL, and aP2) and decreased lipid droplet accumulation. Expression of LRP1 was also shown to be up-regulated in adipose tissues of both obese humans and mice.

We identified a set of 11 genes that were differentially expressed between MFO and MSO at both E16 and E20, despite the marked differences in adipocyte function between these two stages of development. Among these, IGF1R, BMP2, and CD24 have been previously shown to influence adipogenesis in other models. Insulin-like growth factor 1 (IGF-1) plays a crucial role in adipose tissue development and lipid accumulation by upregulating key regulatory genes and signaling through the IGF1R receptor, which is known to be highly expressed in undifferentiated preadipocytes (Boucher et al., 2016; Wang et al., 2019). BMP2 is expressed in adipose progenitor cells and has been shown to promote both adipose hyperplasia and hypertrophy in multiple species (Guiu-Jurado et al., 2016; Blázquez-Medela et al., 2019; Yang et al., 2022). Expression of CD24 serves as a cell surface marker for preadipocytes and is associated with the early stages of adipogenesis, with declining levels of expression in mature adipocytes (Smith et al., 2015). Adipose tissue from MFO also showed consistently higher expression of peptidase M20 domain containing

1 (PM20D1) at E16 and E20. PM20D1 catalyzes synthesis of N-acyl amino acids which interact with mitochondrial iron transporters, specifically members of the SLC25 family, and lead to an increase in proton flux out of the mitochondrial matrix. This effect uncouples oxidative respiration, similar to the effects of UCP1, and (like UCP1) has been shown to enhance cellular energy expenditure (Long et al., 2016; Long et al., 2018a; Kim et al., 2020). To our knowledge, the physiological role of PM20D1 in avians has not been explored. However, it is interesting to speculate that it may play a role in thermogenic capacity of adipocytes in the absence of UCP1, which is not present in avian genomes. Of note, several other genes related to thermogenic adipocytes, including ELOVL3, E2F1, PPARGC1 α , SIRT6, and SLC25A14, showed higher expression in MFO than MSO at E20. Although it was only analyzed ex vivo, we also observed a significant increase in mitochondrial density in adipocytes isolated from MFO vs. MSO at E16. Taken together, these results suggest that MFO may reduce adipocyte lipid accumulation in part by metabolic effects that increase energy expenditure.

At E16, a stage that represents intense adipose tissue expansion, we found that MFO led to an upregulation in the expression of apoptosis-related genes, including fas cell surface death receptor (FAS), NF-KB inhibitor alpha (NFKBIA), and interleukin 15(IL15). DHA has been shown to induce apoptosis by upregulating death receptor expression in cancer cells (Montecillo-Aguado et al., 2023). Furthermore, it is known that death receptors on the cell surface can trigger adipocyte apoptosis (Eguchi and Feldstein, 2014). A previous study by Kim et al. (2006) revealed that DHA induces apoptosis during the early stages of differentiation in 3T3-L1 adipocytes, suggesting that apoptosis-mediated hypertrophic adipocyte death may play a significant role in reducing fat accumulation.

In summary, enriching the hen diet in fish oil leads to alterations in embryo adipose development, accompanied by a substantial deposition of EPA and DHA within the embryo adipose tissue. These changes are characterized by a reduction in adipocyte size, driven by the regulation of genes associated with adipogenesis, lipid metabolism, and mitochondrial function. The findings of this study shed light on the potential impact of maternal fish oil supplementation on embryonic adipose tissue development before hatch. Further research and comprehensive strategies are imperative to fully elucidate the mechanisms through which fish oil in the hen diet influences adipose development in chick embryos. This understanding can be harnessed to explore the potential of maternal fish oil supplementation as an effective and therapeutic approach for mitigating fatness in broiler chickens. In conclusion, in combination with our previous work, these data underscore the significance of developmental programming through the hen diet as a valuable and efficient tool for improving broiler performance in post-hatching.

Appendix

Table 3.1 Ingredient and chemical composition of the experimental diets

Item	MSO	MFO
Ingredient (%)		
Corn	63.2	63.2
Soybean meal (48 %)	11.9	11.9
Wheat midds	8	8
Limestone	7.9	7.9
Pro-pak ¹	5	5
Fish oil	-	2.3
Corn oil	2.3	-
Mono calcium phosphate	0.6	0.6
Termin-8 ²	0.3	0.3
Salt	0.2	0.2
Vit Pre-mix ³	0.2	0.2
Bicarbonate of soda	0.15	0.15
DL-Methionine	0.14	0.14
Choline	0.12	0.12
TM Pre-mix ⁴	0.08	0.08
Chemical composition		
ME, kcal/kg	2938	2938
CP, %	15.2	15.2
Calcium, %	3.5	3.5
Total phosphorus	0.61	0.61
Available phosphorus	0.43	0.43

Abbreviations: MSO, maternal soybean oil; MFO, maternal fish oil.

¹ Protein concentrate, H.J. Baker and Bros., 595 Summer Street, Stamford, CT 06901-1407.

² Antimicrobial preservative.

³ Vitamin premix provided per kg of diet: Vitamin A, 30,800 IU; Vitamin D₃, 9,250 IU; Vitamin E, 153.9 IU; Vitamin B₁₂, 0.154 mg; riboflavin, 46.2 mg; niacin, 185 mg; pantothenic acid, 84 mg; menadione sodium bisulfite, 16.2 mg; folic acid, 12.3 mg; pyridoxine HCl, 46.2 mg; thiamine HCl, 20.5 mg; biotin, 9.3 mg; choline, 2,944 mg; niacin, 185 mg.

⁴ Mineral premix provided per kg of diet: Cu, 55 mg; I, 7.3 mg; Fe, 366 mg; Mn, 310 mg; Zn, 321 mg; K, 2.23 g; Mg, 1.09 g; Se, 0.48 mg.

Table 3.2 Fatty acid composition of the experimental diets

Item¹	MSO	MFO
Capric, C10:0	0.28	0.21
Lauric, C12:0	0.19	0.18
Myristic, C14:0	0.45	2.12
Palmitic, C16:0	15.8	18.1
Stearic, C18:0	15.2	12.8
Oleic, C18:1	10.3	11.7
Linoleic, C18:2n-6 cis	25.9	22.5
Linoleadic, C18:2n-6 trans	23.6	20.5
α -Linolenic, C18:3n-3	1.7	0.85
γ -Linolenic, C18:3n-6	0.15	0.32
Eicosadienoic, C20:2n-6	0.04	0.07
Dihomo- γ -Linolenic, C20:3n-6	0.03	0.05
Eicosatrienoic, C20:3n-3	0.08	0.12
Arachidonic, C20:4n-6	0.02	0.04
Eicosapentaenoic, C20:5n-3	0.15	2.2
Docosadienoic, C22:2n-6	0.02	0.1
Docosahexaenoic, C22:6n-3	0.29	2
Total n-3	2.2	5.2
Total n-6	49.7	43.6
Total fat	5.8	5.8

Abbreviations: MSO, maternal soybean oil; MFO, maternal fish oil.

¹ % of total is the relative percentage of fatty acid methyl esters measured in each sample.

Table 3.3 Fatty acid composition in initial (E0) and residual (E16) yolk

Item ¹	E0		E16	
	MSO	MFO	MSO	MFO
C8:0	0.004 ± 0.0004	0.005 ± 0.0007	0.005 ± 0.0009	0.086 ± 0.079
C10:0	0.001 ± 0.0003 ^{ab}	0.002 ± 0.0004 ^a	0.001 ± 0.0001 ^b	0.002 ± 0.0003 ^{ab}
C12:0	0.01 ± 0.001 ^{ab}	0.01 ± 0.001 ^a	0.005 ± 0.0005 ^b	0.007 ± 0.0004 ^{ab}
C14:0	0.38 ± 0.040 ^{bc}	0.63 ± 0.072 ^a	0.28 ± 0.018 ^c	0.528 ± 0.0323 ^{ab}
C14:1n-5	0.049 ± 0.0129 ^{bc}	0.104 ± 0.016 ^a	0.033 ± 0.0033 ^c	0.078 ± 0.003 ^{ab}
C15:0	0.072 ± 0.005	0.113 ± 0.0158	0.068 ± 0.0049	0.653 ± 0.5417
C15:1n-5	0.003 ± 0.0001 ^{bc}	0.005 ± 0.0004 ^a	0.002 ± 0.0002 ^b	0.003 ± 0.0003 ^b
C16:0 (Palmitic)	28.52 ± 0.923 ^{bc}	31.69 ± 0.884 ^{ab}	27.96 ± 0.33 ^c	32.51 ± 1.05 ^a
C16:1n-7	1.9 ± 0.29 ^{bc}	2.91 ± 0.22 ^a	1.69 ± 0.095 ^c	2.47 ± 0.077 ^{ab}
C17:0	0.13 ± 0.016	0.18 ± 0.02	0.14 ± 0.007	0.31 ± 0.12
C17:1n-7	0.02 ± 0.004 ^{ab}	0.06 ± 0.009 ^a	0.02 ± 0.006 ^b	0.05 ± 0.009 ^{ab}
C18:0 (Stearic)	9.83 ± 0.513 ^{ab}	7.93 ± 0.463 ^{ab}	9.94 ± 0.511 ^a	6.93 ± 0.818 ^b
C18:1n-9c	22.62 ± 0.433	22.12 ± 0.429	23.3 ± 0.461	23.7 ± 0.726
C18:1n-9t	22.55 ± 0.431	22.05 ± 0.427	23.23 ± 0.462	23.63 ± 0.72
C18:2n-6 (LA)	10.79 ± 0.642 ^a	7.67 ± 0.851 ^b	10.56 ± 0.389 ^a	5.89 ± 0.177 ^b
C18:3n-6 (GLA)	0.45 ± 0.018 ^a	0.37 ± 0.046 ^{ab}	0.4 ± 0.015 ^a	0.27 ± 0.014 ^b
C20:0	0.01 ± 0.001	0.01 ± 0.002	0.01 ± 0.002	0.01 ± 0.001
C20:1n-9	0.07 ± 0.004 ^{ab}	0.09 ± 0.004 ^a	0.07 ± 0.004 ^{ab}	0.06 ± 0.003 ^b
C20:2n-6	0.07 ± 0.008 ^a	0.05 ± 0.014 ^{ab}	0.07 ± 0.008 ^a	0.03 ± 0.001 ^b
C20:3n-6 (DGLA)	0.16 ± 0.009 ^a	0.11 ± 0.027 ^{ab}	0.16 ± 0.019 ^a	0.06 ± 0.006 ^b
C20:4n-6 (AA)	1.62 ± 0.081 ^a	0.82 ± 0.278 ^b	1.64 ± 0.142 ^a	0.34 ± 0.019 ^b
C20:5n-3 (EPA)	0.01 ± 0.001 ^b	0.44 ± 0.141 ^a	0.01 ± 0.001 ^b	0.41 ± 0.033 ^a
C22:0	0.009 ± 0.0011	0.006 ± 0.0011	0.007 ± 0.0014	0.0102 ± 0.0009
C22:1n-9	0.0002 ± 0.0001 ^b	0.0017 ± 0.00037 ^a	0.0001 ± 0.00006 ^b	0.0017 ± 0.00013 ^a
C22:6n-3 (DHA)	0.69 ± 0.047 ^b	2.61 ± 0.674 ^a	0.4 ± 0.038 ^b	2.45 ± 0.216 ^a
C24:0	0.007 ± 0.001	0.006 ± 0.001	0.006 ± 0.0007	0.006 ± 0.0007
C24:1n-9	0.006 ± 0.001 ^b	0.012 ± 0.002 ^a	0.006 ± 0.0005 ^b	0.013 ± 0.0016 ^a
ΣSFA	38.97 ± 0.735	40.58 ± 0.632	38.41 ± 0.505	41.05 ± 1.227
ΣMUFA	47.23 ± 0.905	47.35 ± 0.791	48.35 ± 0.889	49.99 ± 1.46
ΣPUFA	13.8 ± 0.769 ^a	12.08 ± 0.601 ^a	13.23 ± 0.477 ^a	9.37 ± 0.355 ^b
Σn-6	13.1 ± 0.722 ^a	9.02 ± 1.205 ^b	12.83 ± 0.455 ^a	6.58 ± 0.18 ^b
Σn-3	0.696 ± 0.047 ^b	3.056 ± 0.807 ^a	0.401 ± 0.039 ^b	2.793 ± 0.265 ^a
Σn-6/Σn-3	18.89 ± 0.352 ^b	7.91 ± 3.942 ^{bc}	33.27 ± 2.829 ^a	2.44 ± 0.199 ^c

Values are expressed as mean ± SEM (n = 6).

^{a,b,c} Means within a row with different superscripts differ significantly ($P < 0.05$).

Abbreviations: MSO, maternal soybean oil; MFO, maternal fish oil; SFA, saturated fatty acids;

MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids.

¹ % of total is the relative percentage of fatty acid methyl esters measured in each sample.

Table 3.4 Fatty acid composition in developing (E16) adipose tissue

Item¹	MSO	MFO	P-value
C4:0	0.007 ± 0.0007	0.006 ± 0.0004	0.405
C6:0	0.002 ± 0.001	0.001 ± 0.0004	0.463
C8:0	0.006 ± 0.0005	0.006 ± 0.0004	0.998
C10:0	0.019 ± 0.0019	0.015 ± 0.0011	0.088
C12:0	0.084 ± 0.0067	0.08 ± 0.002	0.549
C13:0	0.003 ± 0.0003	0.004 ± 0.0002*	0.001
C14:0	0.92 ± 0.0432	1.104 ± 0.0304*	0.007
C14:1n-5	0.113 ± 0.0065	0.128 ± 0.0043	0.087
C15:0	0.09 ± 0.0064	0.128 ± 0.0045*	0.001
C15:1n-5	0.005 ± 0.0005	0.006 ± 0.0004	0.055
C16:0 (Palmitic)	30.45 ± 0.386	31.86 ± 1.031	0.246
C16:1n-7 (Palmitoleic)	1.71 ± 0.091	2.14 ± 0.093*	0.008
C17:0	0.15 ± 0.013	0.19 ± 0.004	0.034
C17:1n-7	0.03 ± 0.004	0.06 ± 0.004*	0.001
C18:0 (Stearic)	6.33 ± 0.194	5.8 ± 0.153	0.06
C18:1n-9c	21.77 ± 0.422	22 ± 0.352	0.694
C18:1n-9t	21.73 ± 0.421	21.96 ± 0.351	0.691
C18:2n-6 (LA)	12.8 ± 0.776	7.27 ± 0.384*	<0.001
C18:3n-6 (GLA)	0.47 ± 0.037	0.25 ± 0.014*	0.001
C20:0	0.02 ± 0.001	0.02 ± 0.001	0.803
C20:1n-9	0.1 ± 0.006	0.09 ± 0.001	0.066
C20:2n-6	0.1 ± 0.008	0.04 ± 0.002*	<0.001
C20:3n-6 (DGLA)	0.18 ± 0.008	0.09 ± 0.004*	<0.001
C20:4n-6 (AA)	1.52 ± 0.096	0.31 ± 0.016*	<0.001
C20:5n-3 (EPA)	0.08 ± 0.01	0.78 ± 0.028*	<0.001
C21:0	0.0008 ± 0.0002	0.0003 ± 0.00006*	0.032
C22:0	0.008 ± 0.0006	0.007 ± 0.0004	0.115
C22:1n-9	0.0036 ± 0.0003	0.003 ± 0.0002	0.172
C22:6n-3 (DHA)	1.29 ± 0.134	5.64 ± 0.330*	<0.001
C24:0	0.002 ± 0.0003	0.002 ± 0.0003	0.18
C24:1n-9	0.005 ± 0.0004	0.005 ± 0.0004	0.861
ΣSFA	38.10 ± 0.239	39.23 ± 1.04	0.334
ΣMUFA	45.48 ± 0.922	46.39 ± 0.751	0.463
ΣPUFA	16.43 ± 0.99	14.39 ± 0.505	0.107
Σn-6	15.06 ± 0.895	7.97 ± 0.393*	<0.001
Σn-3	1.37 ± 0.144	6.42 ± 0.345*	<0.001
Σn-6/Σn-3	11.5 ± 1.025	1.26 ± 0.095*	<0.001

Table 3.4 continued

Activity index²			
SCD 16	0.06 ± 0.003	0.07 ± 0.004*	0.042
SCD 18	6.91 ± 0.253	7.61 ± 0.288	0.097
D5D	8.53 ± 0.388	3.69 ± 0.136*	<0.001
D6D	0.04 ± 0.001	0.03 ± 0.001	0.224
DNL	2.43 ± 0.167	4.48 ± 0.398*	0.002
EL	0.21 ± 0.009	0.18 ± 0.007*	0.047

Values are expressed as mean ± SEM (n = 6).

* $P < 0.05$ by Student's t-test.

Abbreviations: MSO, maternal soybean oil; MFO, maternal fish oil; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; SCD, Stearoyl-CoA desaturase; D5D, Delta-5 desaturase; D6D, Delta-6 desaturase; DNL, De novo lipogenesis; EL, Elongation.

¹ % of total is the relative percentage of fatty acid methyl esters measured in each sample.

² Enzyme activities were calculated using the following equations: SCD16, 16:1n-7/16:0; SCD18, 18:1n-9/18:0; D5D, 20:4n-6/20:3n-6; D6D, 18:3n-6/18:2n-6; DNL, 16:0/18:2n-6; EL, 18:0/16:0.

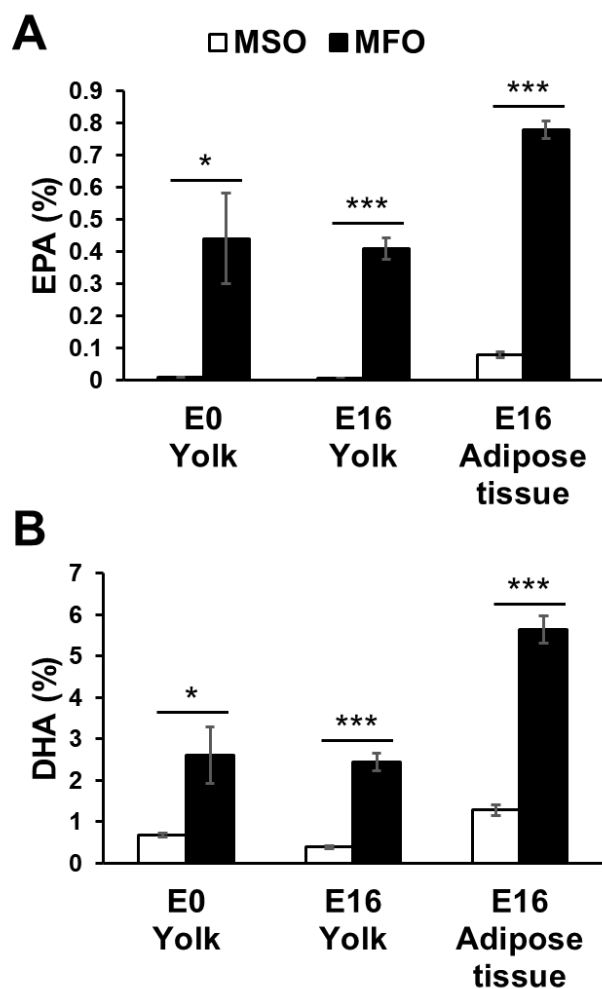


Figure 3.1 Effect of MFO on EPA and DHA contents in yolk and developing adipose tissue of broiler chick embryo

Comparison of (A) EPA and (B) DHA contents in yolk (E0 and E16) and E16 adipose tissue between MSO and MFO. Values are expressed as mean $\pm$ SEM (n = 6). * P < 0.05, *** P < 0.001 by Student's t-test.

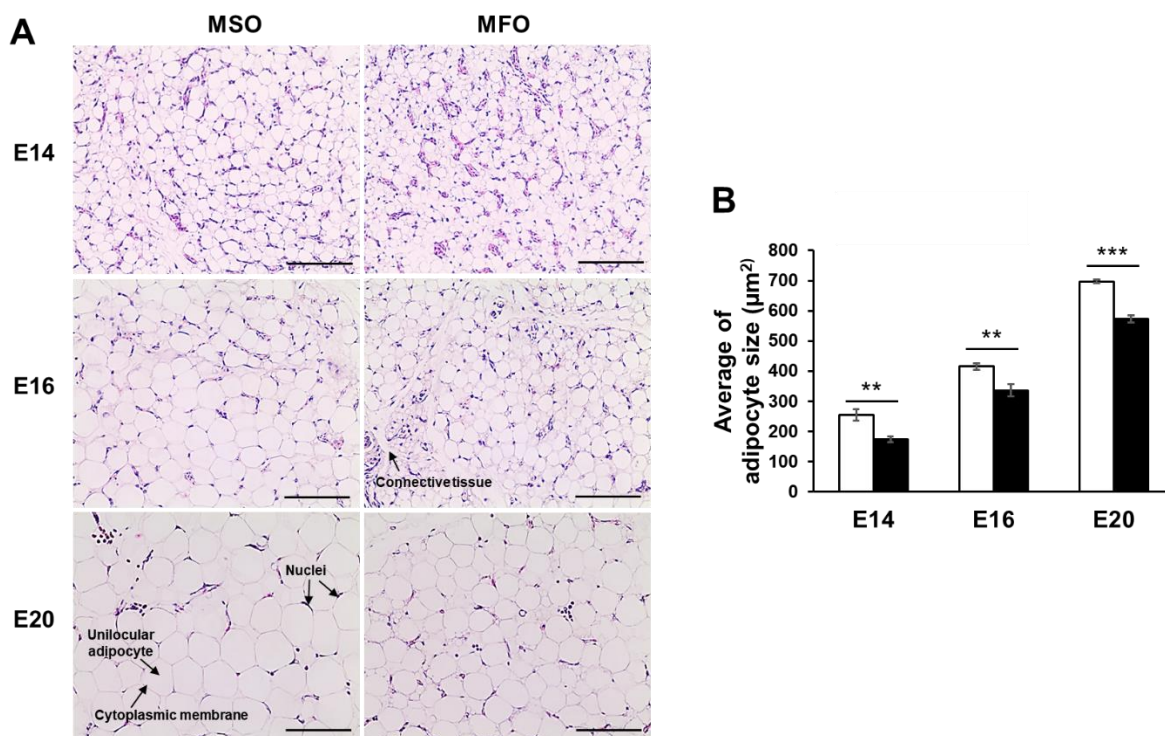


Figure 3.2 Effect of MFO on adipose tissue growth and development of broiler chick embryo

(A) Hematoxylin-eosin (H&E) stained sections of subcutaneous adipose tissue at E14, E16, and E20. Scale bars = 100 μm . (B) Comparison of average adipocyte size between MSO and MFO. Values are expressed as mean $\pm$ SEM ($n = 5$). ** $P < 0.01$, *** $P < 0.001$ by Student's t-test.

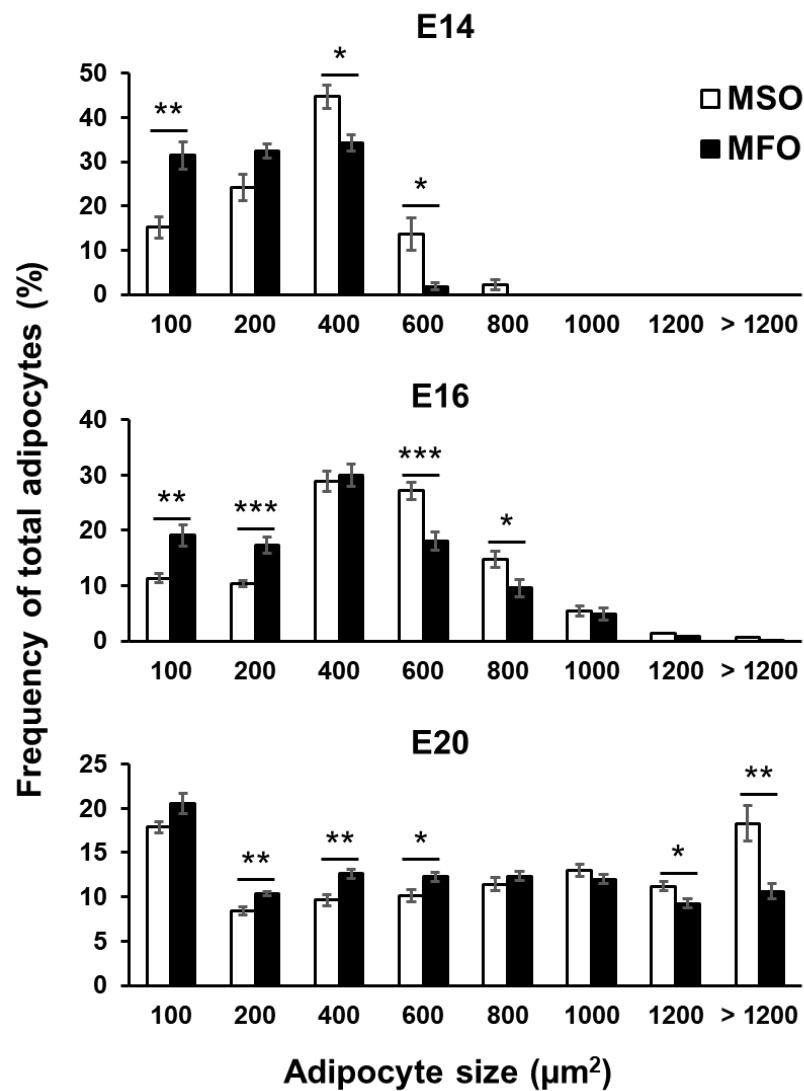


Figure 3.3 Comparison of the frequency distribution of adipocyte size between MSO and MFO at E14, E16, and E20

Values are expressed as mean $\pm$ SEM ($n = 5$). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ by Student's t-test.

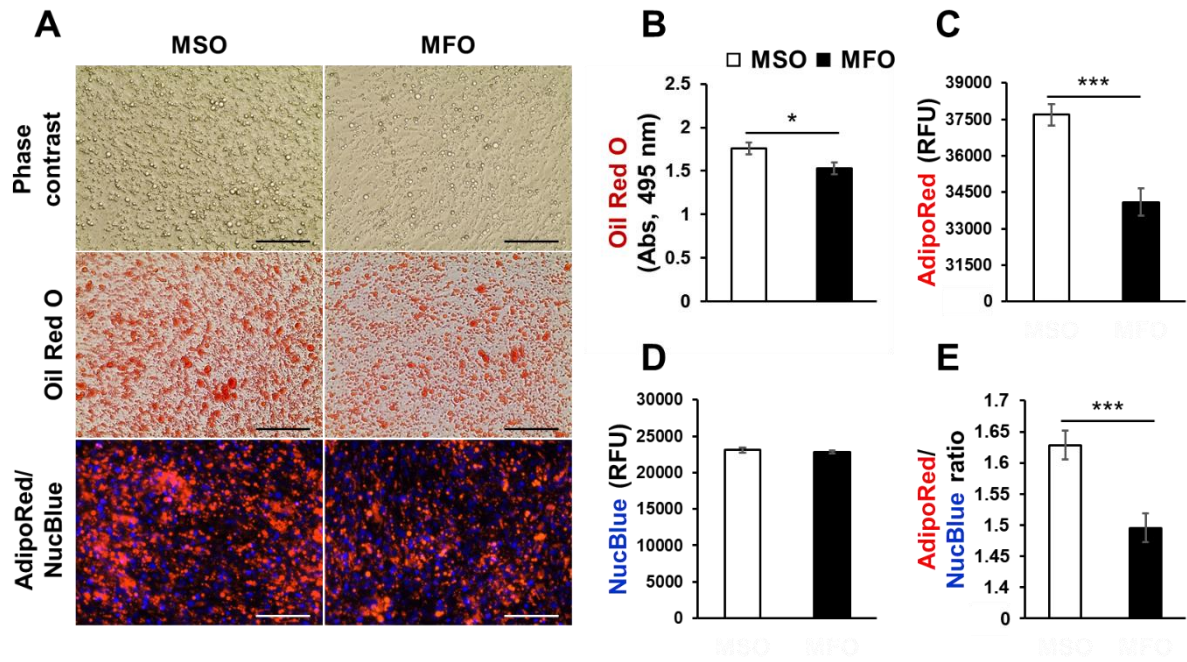


Figure 3.4 Effect of MFO on lipid accumulation in preadipocytes isolated from subcutaneous adipose tissue of broiler chick embryo at 72 h differentiation

(A) Representative images of phase contrast, lipid staining (red), and DNA staining (blue) of preadipocytes from MSO and MFO. Scale bars = 100 μ m. (B) Quantification of lipid droplets were measured by elution of Oil Red O staining. (C) AdipoRed and (D) NucBlue staining were measured by detecting fluorescent probes within the cells. (E) Lipid accumulation was normalized to DNA content. Values are expressed as mean $\pm$ SEM (n = 3). * P < 0.05, *** P < 0.001 by Student's t-test.

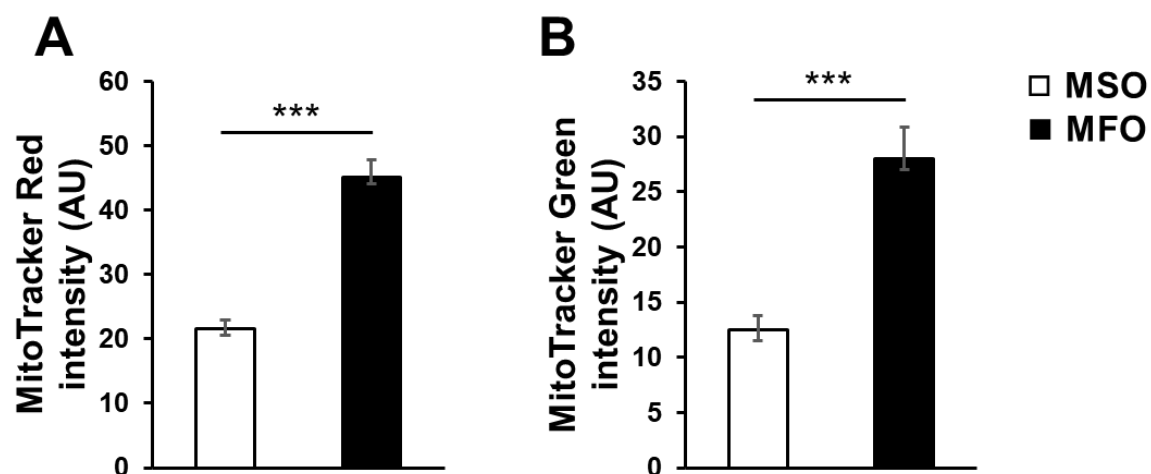


Figure 3.5 Effect of MFO on mitochondrial function in preadipocytes isolated from subcutaneous adipose tissue of broiler chick embryo

(A) Mitochondrial activity and (B) mitochondrial content were measured by detecting fluorescent intensity of MitoTracker Red and Green, respectively. Values are expressed as mean $\pm$ SEM (n = 3). *** $P < 0.001$ by Student's t-test.

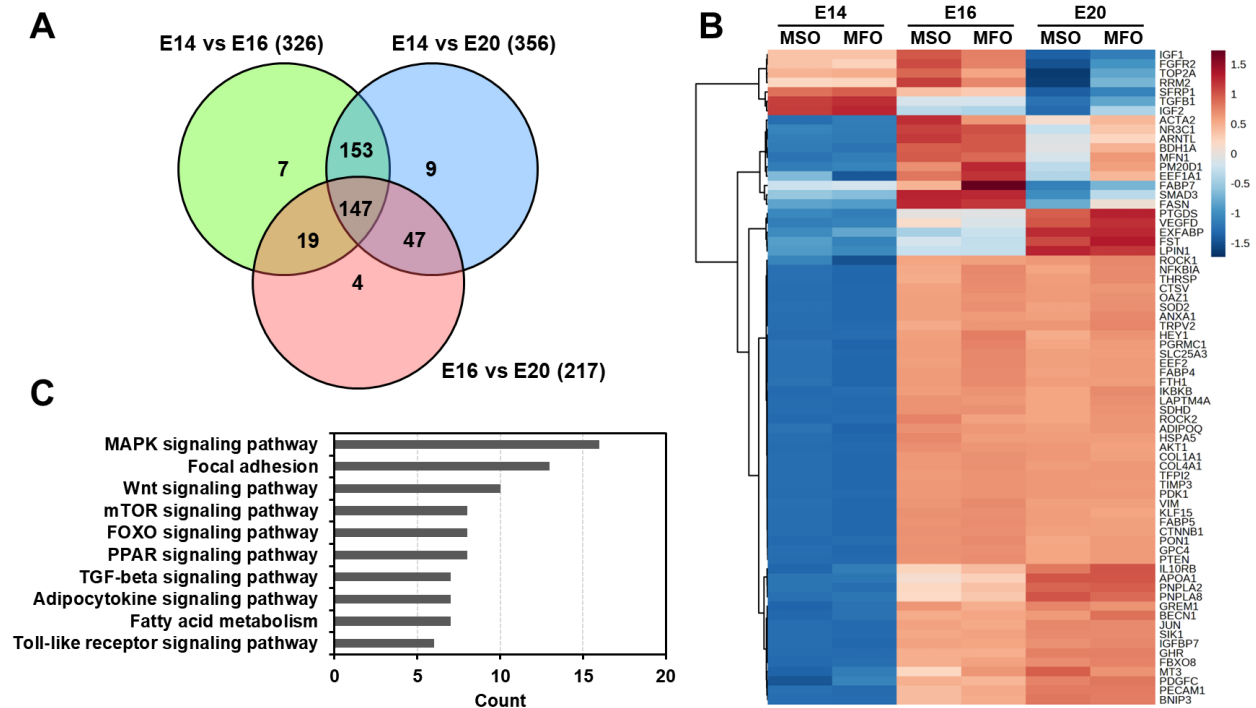


Figure 3.6 Differentially expressed genes across embryonic ages (FDR $P < 0.05$)

(A) Venn diagram illustrating comparisons among three age groups. (B) Heatmap showing the top 70 genes from the 147 overlapping DEGs identified in the Venn diagram. (C) Top 10 significantly enriched KEGG pathways of the 147 genes.

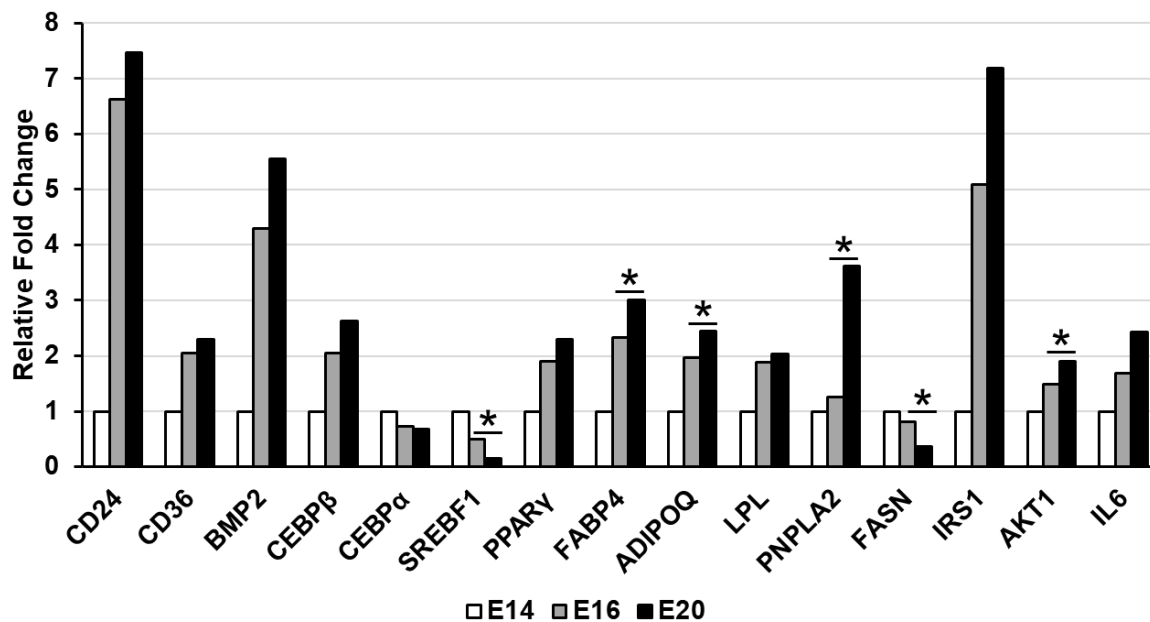


Figure 3.7 Comparison of gene expression associated with adipose development across embryonic ages

The bar graph shows fold change relative to E14 at E16 and E20. The selected genes represent makers of adipocyte progenitors (CD24, CD36), differentiating preadipocyte (BMP2, CEBPβ, CEBPα, SREBF1, PPARγ), mature adipocyte (FABP4, ADIPOQ, LPL, PNPLA2, FASN), insulin signaling (IRS1, AKT1), and adipocyte inflammation (IL6). All the selected markers were significantly different at E16 and E20 compared to E14 (FDR $P < 0.05$). * E16 vs. E20 (FDR $P < 0.05$).

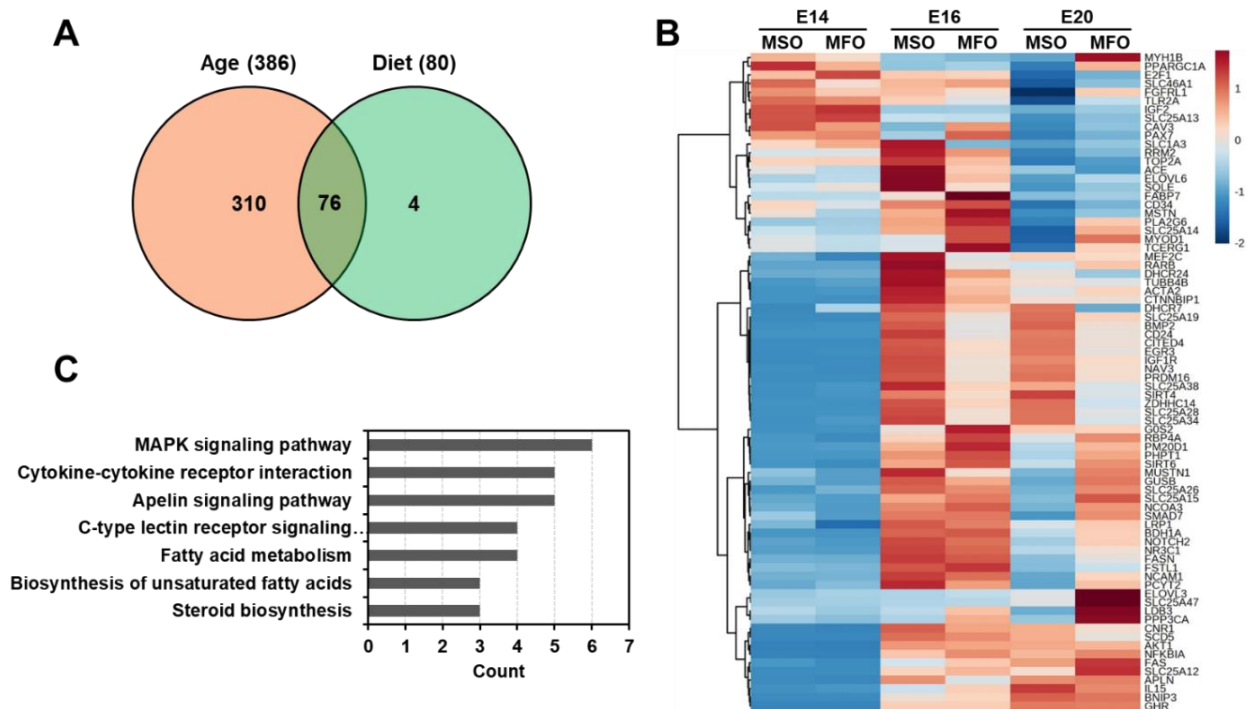


Figure 3.8 Effect of MFO on molecular profiling of chick embryo adipose tissue

(A) Venn diagram of overlapping DEGs identified by embryonic age and hen diet (FDR $P < 0.05$). (B) Heatmap of 76 overlapping genes obtained from Venn diagram. (C) KEGG pathway enrichment analysis of the 76 genes.

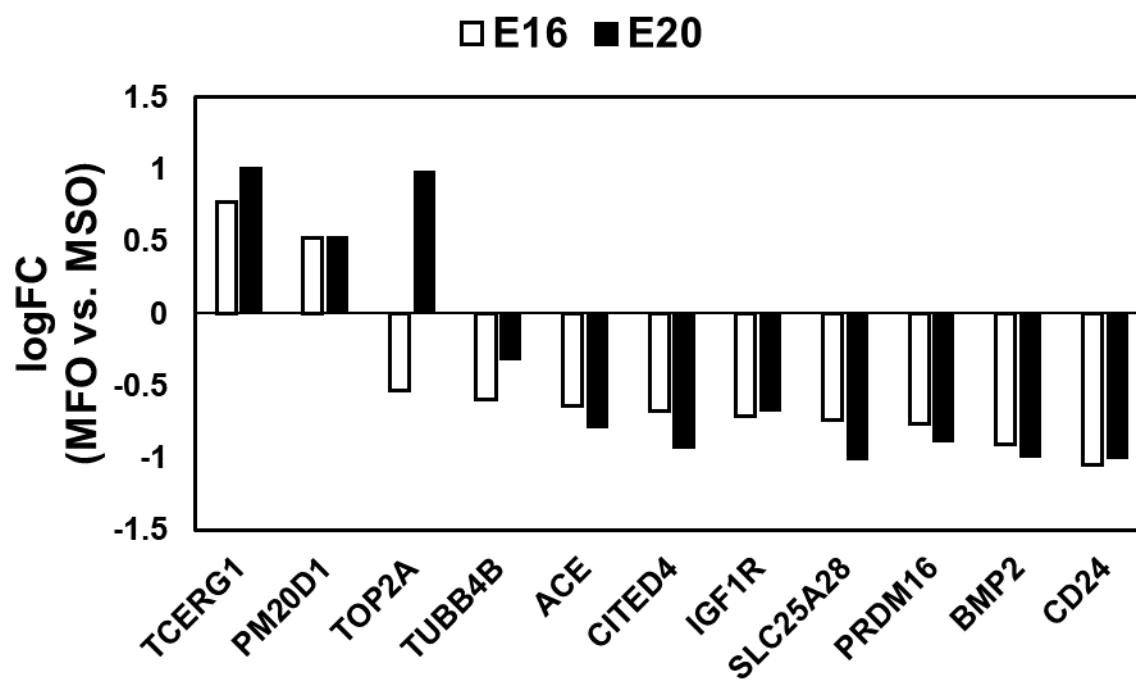


Figure 3.9 Bar plot of log₂ fold change (logFC) of the common DEGs between MSO and MFO adipose tissue at E16 and E20 (FDR $P < 0.05$)

Positive values indicate up-regulation in MFO, while negative values indicate down-regulation in MFO vs. MSO.

CHAPTER 4

EFFECTS OF MATERNAL FISH OIL ON TRANSCRIPTOME AND METHYLOME IN ADIPOSE TISSUE IN BROILER CHICKE EMBRYO

A version of this chapter is being prepared for publication in *Poultry Science* by Minjeong Kim and Brynn Voy.

Abstract

Dietary programming can produce lasting effects on growth and metabolism through epigenetic mechanisms that persist across generations. We previously demonstrated that providing the developing broiler chick embryo with omega-3 polyunsaturated fatty acids (**n-3 PUFA**) found in fish oil attenuated adipose development and reduced adiposity in chicks after hatch. Our objective in this study was to identify transcriptomic and epigenomic changes that may be responsible for these effects. Ross 708 broiler breeder hens (n=30/diet) were fed diets containing 2.3 % fat from fish oil or soybean oil (n-6 PUFA control) for four weeks. Fertilized eggs from the maternal fish oil (**MFO**) group or soybean oil group (**MSO**) were randomly collected and incubated until embryonic age 16. We extracted RNA and DNA from adipose tissues in the same embryos and used for genome-wide RNAseq analysis and methylated DNA immunoprecipitation sequencing (MeDIP-seq), respectively. Expression levels of 323 genes were significantly influenced by MFO, with 210 upregulated and 113 down-regulated genes compared to MSO control (FDR $P < 0.05$). Differentially expressed genes included transcription factors known to control tissue development as well as genes involved in lipid metabolism. Additionally, MFO increased the expression of four members of the Iroquois homeobox genes, which have recently been shown to regulate adipogenesis in humans and rodents. The type of fatty acids in the hen diet also significantly influenced patterns of DNA methylation. A total of 12 differentially methylated regions (**DMRs**) were identified (FDR $P < 0.05$), with 10 of these DMRs exhibiting hypomethylation in MFO vs. MSO, and two unique genes were annotated. In conclusion, MFO influenced adipose development

in chick embryos through transcriptomic and epigenetic modifications to the genome. Building upon our previous findings, this data further supports the potential mechanisms by which MFO stably modifies adipose accretion in broiler embryo and post-hatch chick.

Introduction

Broiler chickens are incredibly efficient for meat production due to intensive genetic selection for rapid growth and muscle yield over several decades. However, selection inadvertently enhanced accretion of adipose tissues in broilers (Siegel, 2014; Zuidhof et al., 2014; Moreira et al., 2018). As a result, broiler chickens have a genetic tendency to accumulate excessive fat by utilizing feed that could otherwise be used for primary meat production. This poses a challenge for poultry producers as it leads to depressed feed efficiency, which is the highest component of production cost, and thus reduced profitability. In broiler-breeder hens, the accumulation of excess lipids impacts their reproductive efficiency, resulting in substantial losses in production revenue. Given the scale of broiler production and the growing concern for animal welfare, the development of efficient tools to mitigate excess adipose accumulation in broilers will become increasingly important.

Dietary nutrients, particularly dietary fat sources, have a significant impact on the development and growth of adipose tissue. Omega-3 polyunsaturated fatty acids (**n-3 PUFA**) have been shown to have anti-obesogenic effects in vitro, and in a number of species in vivo. This class of fatty acids can attenuate lipid accumulation by regulating genes associated with adipogenesis and promoting fatty acid oxidation, lipolysis, and adipose thermogenesis (Crespo and Esteve-Garcia, 2002b; c; Azain, 2004; Todorčević and Hodson, 2015; Chen et al., 2020; Zapata et al., 2022; Raj et al., 2023). Eicosapentaenoic acid (**EPA**; C20:5n-3) and docosahexaenoic (**DHA**;

C22:6n-3) acids are the main contributors to the anti-obesogenic effects of n-3 PUFA. Previous studies have demonstrated that supplementing feed with fish oil, either alone or in combination with other n-3 PUFA sources, can improve body composition by inhibiting adipose tissue development and reducing fat accumulation in chickens (Gonzalez-Ortiz et al., 2013; Torchon et al., 2017; Ibrahim et al., 2018; Kim and Voy, 2021). A recent study reported that enriching the broiler diet with fish oil (at 0.05%, 0.1%, and 0.15%) for 3 weeks increased breast muscle weight and decreased abdominal fat content when compared to the control group (Wahid et al., 2023).

Our lab has previously shown that providing n-3 PUFA to the developing chick embryo, through the diet of the hen, is a potential means to attenuate excess fat deposition in broilers. Chicks hatched from hens fed a fish oil-enriched diet showed lower body fat percentage at 7 and 14 days compared to those hatched from hens fed a corn oil-based diet, with no effects on lean growth or breast muscle weight (Beckford et al. (2017). These effects on adipose development were associated with significant reductions in adipocyte size, and differential expression of genes and proteins involved in adipogenesis, fatty acid transport, and lipid metabolism in the adipose tissue of post-hatch chicks. The potential benefits of n-3 PUFA on chick development may persist to market age and result in sustained impact on traits that benefit production. Recently, we demonstrated that enriching the hen diet in fish oil enhanced the growth performance and skeletal integrity of chicks raised to 49d of age (Tompkins et al., 2022). This enhancement resulted in increased lean mass, bone mineral content, and reduced porosity volume in the tibia when compared to the control group, in which hens were fed diets containing an equivalent amount of soybean oil. Effects on bone integrity were associated with reduced expression of adipogenic genes, including PPAR γ , FABP4, and CEBP β , in the bone marrow as they reached market age. These sustained effects are consistent with other studies in which broiler-breeder hen diets

enriched with n-3 PUFA have been shown to improve immunity, gut microbiota, skeletal development, and cardiac function in offspring (Bautista-Ortega et al., 2009; Cherian, 2011; Akbari Moghaddam Kakhki et al., 2021; Thanabalan and Kiarie, 2021; Tompkins et al., 2022). Taken together, these findings suggest that enriching the hen diet with fish oil has the potential to effectively program reduced fat accumulation in post-hatch chicks by modulating adipogenesis and lipid metabolism during the embryonic development stage. However, there is still a knowledge gap regarding the impact of these fatty acids present in the yolk on adipose development, metabolism, and body composition in chick embryos. Filling this gap is crucial for developing practical and effective ways to limit adiposity and promote lean growth in broiler chickens.

Persistent effects of the hen diet on adipose development may result from epigenetic modifications to the chick genome that influence gene expression in ways that reduce fat accumulation. Maternal environmental factors, such as stress, nutrition, or obesity during embryonic development, can lead to long-lasting phenotypic changes through inheritable epigenetic mechanisms that regulate gene expression. These effects occur in part through epigenetics, which serves as the critical interface where environmental factors and the genome interact. Epigenetic processes include DNA methylation, histone acetylation, and the regulation of noncoding RNA. Among these mechanisms, DNA methylation is currently the most extensively studied epigenetic mark. This process involves the addition of small chemical groups (methyl or hydroxymethyl groups) to the DNA sequence, primarily within regions known as cytosine-phosphate-guanosine (CpG) dinucleotides (Gardiner-Garden and Frommer, 1987). Notably, DNA methylation acts as a stable and heritable epigenetic modification, and its primary role lies in the regulation of gene expression without altering the DNA sequence. In particular, DNA methylation at CpG sites in gene promoters directly influences gene expression by altering DNA conformation

and, consequently, the accessibility of transcription factors (Zhu et al., 2016). Hypermethylation typically represses gene transcription, while normal cells typically exhibit an unmethylated (hypomethylated) state associated with gene expression. These epigenetic alterations can persist throughout the entire life of the organism, as this modification is retained during cell divisions (Feil and Fraga, 2012). Notably, a recent study by Li et al. (2021) demonstrated that maternal supplementation with n-3 PUFA during pregnancy increased DNA methylation levels at specific CpG sites within obesity-related genes, such as PPAR γ , CEBP α , adiponectin, IGF-2, and leptin. These changes may potentially reduce obesity-related gene expression in infants.

DNA methylation is an essential process that occurs during embryogenesis, guiding gene expression and shaping an organism's development. During embryogenesis, DNA methylation patterns are established and modified in a highly controlled manner (Razin and Cedar, 1993). Consequently, these epigenetic marks are particularly sensitive to environmental influences during this critical embryonic phase (Skinner, 2011; Feil and Fraga, 2012). For instance, maternal heat stress during fetal development can affect offspring performance through epigenetic programming. Exposure to intrauterine heat stress during gestation altered the DNA methylation profile and gene expression of bull calf livers (Skibieli et al., 2018). Moreover, Akbarinejad et al. (2017) reported that maternal heat stress during gestation has detrimental effects on fertility, productive longevity, and ovarian reserve of female bovine offsprings. Furthermore, during the maturation of bovine oocytes, exposure to high levels of non-esterified fatty acids, a common factor associated with maternal obesity, resulted in a diminished capacity to develop into blastocysts that resulted from alterations in DNA methylation and transcriptomic profiles compared to control counterparts (Desmet et al., 2016). Hence, the ability to influence embryo development through the maternal environment holds considerable significance in the context of production animals.

Genome-wide DNA methylation patterns in chicken have been characterized in various tissues, including muscle, liver, retina, and brain, suggesting its important role in chicken development (Li et al., 2011; Hu et al., 2013; Liu et al., 2016; Lee et al., 2017; Vinoth et al., 2018; Liu et al., 2019; Zhang et al., 2023). For instance, DNA methylation has been linked to differences in the rate of embryonic muscle development in layer vs. broiler chickens (Liu et al., 2019). A more recent study by Ran et al. (2021) also investigated genome-wide DNA methylation and transcriptomes of muscle tissues in chicken embryos. This study revealed that differentially methylated genes were closely associated with muscle development, and a negative correlation was observed between gene expression and methylation in promoter regions. These findings indicate that DNA methylation plays a pivotal role in early muscle development, affecting muscle performance by regulating gene expression. Changes in DNA methylation patterns have also been associated with lasting impact on the thermotolerance capacity of chickens. Chicks hatched from eggs exposed to higher incubation temperatures, when subjected to heat stress conditions, displayed elevated methylation levels in the promoter regions of heat-induced stress genes, enhancing the offspring's ability to tolerate stress conditions.

Collectively, the potential of n-3 PUFA to regulate offspring adiposity and metabolism during embryonic development holds significant implications for poultry production. The objective of this study was to discover the effects of enriching tissues of the developing chick embryo with fish oil, as a source of EPA and DHA, on adipose tissue development at the molecular level. We tested the hypothesis that fish oil influences expression of genes and pathways that regulate adipose development. Specifically, we investigated the differential effects of fish oil and soybean oil, as an n-6 PUFA control, on genome-wide gene expression and on DNA methylation in adipose tissue of chick embryos at E16.

Materials and methods

Enriching the Embryo in EPA and DHA

Experimental animals were prepared using the same procedures and formulations, as previously described (Beckford et al., 2017). Briefly, Ross 708 broiler eggs were collected from hens that are fed standard broiler-breeder diets containing 2.3 % (wt:wt) of either soybean oil (**SO**; Conagra Brands, Chicago, IL) or fish oil (**FO**; Jedwards International, Braintree, MA) ad libitum for 4 weeks. This source of FO contains 18% EPA and 12% DHA. Fertilized eggs from the maternal fish oil (**MFO**) group or soybean oil group (**MSO**) were then incubated under standard conditions until embryonic ages (**E**) 16.

Adipose Tissue Collection

Embryos (n=10/diet) were euthanized by cervical dislocation and dissected to collect adipose tissue. Weights of embryo and fat pad were measured. The pair of femoral adipose depots was collected from each chick and snap-frozen in liquid nitrogen. Before storage at -80°C , each pair of fat pads was pulverized under liquid nitrogen to produce a homogeneous powder for both RNA and DNA isolation.

RNA Sequencing Analysis

Approximately 25 mg of pulverized adipose tissue was used for isolation of total RNA and cDNA synthesis as previously described in chapter 3. After verifying RNA quality and integrity, total RNA samples were sent to the Molecular Genomics Core, Texas A&M Institute for Genome Sciences and Society (College Station, TX) for library construction and sequencing. Libraries were prepared using NEBNext Ultra RNA Library kit (Illumina, San Diego, CA) and sequencing was

performed using Illumina Hi-Seq 4000 sequencing platform (Illumina, San Diego, CA) according to the manufacturer's instructions. A minimum of 20M PE150 reads were produced from each RNA sample. Raw sequencing reads were processed by High Performance Computing in Biology group at University of Illinois (Urbana, IL). Briefly, quality of sequence data was evaluated using FASTQC (v 0. 11. 8) on individual samples then was summarized into a single html report by using MultiQC (version 1.9). Reads were trimmed to remove adapters using Trimmomatic3 (v 0.38), then performed FASTQC and MultiQC again. The cleaned reads were then aligned to the chicken genome (bGalGal1.mat.broiler.GRCg7b, NCBI, Annotation 105) using Salmon (v1.4.0). The TMM (trimmed mean of M values) normalization in the edgeR package was performed and normalized log2-based count per million values (logCPM) were calculated using edgeR (McCarthy et al., 2012) to help stabilize fold-changes of extremely low expression genes. After normalization, remove Unwanted Variation (RUV) was used to remove effects of correlated data structures (Peixoto et al., 2015). Differentially expressed genes (DEGs) were identified using the limma-trend method (Ritchie et al., 2015; Chen et al., 2016b) to find the differences between MFO and MSO samples, and multiple testing correction was done using the False Discovery Rate (FDR) method (Benjamini and Hochberg, 1995).

DNA Methylation Analysis

Genomic DNA was isolated from same adipose tissue used in RNAseq. Approximately 25 mg of pulverized adipose tissue was used for isolation of DNA using a DNeasy Blood & Tissue kit (69504, Qiagen, Hilden, Germany), and each DNA sample was quantitated fluorometrically using a Qubit dsDNA BR assay kit (Q32850, Invitrogen, Waltham, MA). Methylation analyses were performed at the SNP & SEQ facilities of the SciLifeLab (Uppsala, Sweden). The integrity of

isolated DNA was evaluated by agarose gel electrophoresis. Genomic DNA was sonicated (six cycles of 30'' ON and 90'' OFF using a Bioruptor Plus sonication device (Diagenode, Liege, Belgium)) to yield fragment sizes of ~450 bp. Approximately 2.7µg of size-selected gDNA from each sample was subjected to immunoprecipitation using by an anti-methyl-cytosine antibody (Diagenode, Sparta, TN). Libraries of methylated DNA were prepared using the ThruPLEX DNA-Seq kit (Takara) according to the manufacturer's instructions. Libraries were quantified, clustered, and paired-end sequenced in the Illumina NovaSeq6000 platform using flowcell kit (S Prime v1.5) with a read length of 150bp.

Raw paired-end sequencing data were preprocessed using TrimGalore (v 0.6.1) to remove adapter sequences and low-quality reads, followed by alignment to the chicken reference genome (*Gallus_gallus.bGalGal1.mat.broiler.GRCg7*) using Bowtie2 (v 2.3.5.1). Resulting SAM files were converted to BAM format and sorted using Samtools (v 0.1.19), with subsequent indexing for efficient downstream analysis. Aligned data were further processed to calculate read depth statistics for each sample. To investigate differential DNA methylation, we renamed and organized the sorted BAM files and applied MACS2 peak calling software was used to identify regions of interest, considering broad peaks with a cutoff of 0.05 for statistical significance. This analysis was performed separately for two experimental conditions, and the results were visualized and interpreted to uncover potential epigenetic regulatory elements associated with the biological context under investigation. In the R-based analysis, we utilized the "BSgenome.Ggallus.NCBI.galGal7" genome as a reference. The main R packages used for data analysis and visualization included MeDIPS for data processing and differential methylation analysis, ChIPseeker for gene annotation using the "Gallus gallus" database. Genomic distribution of differentially methylated regions was assigned on the following criteria: promoter regions

defined as 1kb, 1-2kb, and 1-3kb from transcription start site (TSS), 3' and 5' untranslated regions (UTR), exons, introns, the downstream regions of gene end, and distal intergenic regions.

Functional Enrichment and Interaction Network Analysis

Gene Ontology (GO) annotation and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analyses were performed using the Database for Annotation, Visualization and Integrated Discovery (DAVID, v2023q2). To explore the relationship among the genes of interest, the Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) database (<https://string-db.org/>, version 12.0) was used to generate interaction network structures for the selected genes with default settings.

Results

Effect of MFO on E16 Adipose Transcriptome

Transcriptome profiling by RNAseq in E16 adipose tissue revealed significant differences between MSO and MFO. A total of 323 genes were significantly influenced by MFO (FDR $P < 0.05$), with 210 upregulated and 113 down-regulated genes compared to MSO control (Supplementary Table 1 and 2). The heatmap in Figure 4.1A illustrates the expression patterns of these 323 DEGs in E16 adipose tissue between MSO and MFO. To investigate functional characteristics of these genes, GO terms and KEGG pathway enrichment analysis was performed. The representative GO terms and KEGG pathways are shown in Figure 4.1B, and Table 4.1 lists corresponding genes based on their expression levels in MFO. The GO analysis revealed that DEGs were mainly enriched in biological processes related to embryo development, cell proliferation, apoptotic and metabolic processes. Additionally, they showed enrichment in plasma membrane, cellular structures, and

transcription activity, based on the classification of cellular composition and molecular function. In the KEGG pathway analysis, the up-regulated genes in MFO were more enriched in pathways, including endocytosis, tight junctions, calcium signaling, fatty acid metabolism, the PPAR signaling pathway, and the adipocytokine signaling pathway.

Four members of the Iroquois homeobox (IRX) genes, which have recently been shown to regulate adipogenesis in humans and rodents, were expressed at significantly higher levels in MFO vs. MSO (Figure 4.2). Therefore, we focused on these four IRX genes and the 16 lipid metabolism genes identified through enrichment analysis for further investigation (Table 4.2). Along with the four IRX genes, 13 genes were significantly up-regulated by MFO including stearoyl-CoA desaturase (SCD), cytochrome P450 family 26 subfamily B member 1 (CYP26B1), neuropeptide Y (NPY), cellular retinoic acid binding protein 1 (CRABP1), leptin receptor (LEPR), acyl-CoA synthetase long-chain family member 6 (ACSL6), fatty acid binding protein 7 (FABP7), twist family bHLH transcription factor 1 (TWIST1), fibroblast growth factor receptor 3 (FGFR3), ELOVL fatty acid elongase 7 (ELOVL7), acyl-CoA synthetase long chain family member 3 (ACSL3), acyl-CoA synthetase bubblegum family member 2 (ACSBG2), and 3-hydroxyacyl-CoA dehydratase 3 (HACD3). The expression of 3 genes, patatin like phospholipase domain containing 3 (PNPLA3), malic enzyme 1 (ME1), and FGR proto-oncogene, Src family tyrosine kinase (FGR) was significantly down-regulated in MFO adipose tissue.

We explored evidence for potential interactions among the protein products of IRX family members and DEGs related to lipid metabolism using the STRING protein-protein interaction network. As shown in Figure 4.3, IRX1, IRX2 and IRX3 are associated with TWIST1, based on co-expression and text-mining evidence. Both TWIST1 and IRX3 are associated with a larger set of established interactions among genes in this set that regulate fatty acid activation, desaturation,

elongation, and metabolism. In combination, these putative interactions support a potential role for IRX family members, particularly IRX3, in regulation of lipid metabolism.

Effect of MFO on E16 Adipose Methylome

Genome-wide DNA methylation analysis was conducted to investigate the impact of maternal fatty acid programming on epigenetic modifications to the genome. Methylated sequences captured by MeDIP mapped to 30501 regions on 39 chromosomes. These sequences were located within promoter regions (3kb from TSS, 33%), 5' UTR (0.52 %), 3' UTR (4.04%), exons (12.07%), introns (39.71%), downstream of genes (0.12%), and distal intergenic regions (22.4%). The type of fatty acids in the hen diet significantly influenced DNA methylation patterns, including regions that were hyper- and hypo-methylated in MFO vs. MSO. The numbers of differentially methylated regions (**DMRs**) between MSO and MFO based on different levels of significance are presented in Table 4.3. As shown in Figure 4.4, differential methylation skewed towards hypomethylation in MFO, with 87 hypo-methylated and 26 hyper-methylated regions (FDR $P < 0.3$). With a more stringent threshold (FDR $P < 0.05$), we identified 12 regions that were differentially methylated in MFO vs. MSO (Table 4.4). The distribution of the 12 DMRs in relation to chromosomal locations is presented in Figure 4.5. These DMRs were found on Chr1 (4), Chr 2 (1), Chr 3 (1), Chr8 (1), Chr16 (1), Chr 22 (1), Chr 36 (1), and ChrW (2). Methylation sites were most likely to be associated within a promoter region, located within 1kb upstream or downstream of the transcription start site (50%), followed by distal intergenic regions (25%), and introns (25%) (Figure 4.6). The 12 DMRs were annotated to identify the known gene in closest proximity to the methylation site, as the most closely associated transcriptional target based on chromosomal position. For seven of the 12 DMRs, this identified genes specifying long non-coding RNAs

(LncRNAs) rather than protein-coding genes. Two DMRs reside within the structure of known genes. A region within an intron of kinesin family member 14, KIF14 (Chr8) was hypermethylated in MFO vs. MSO, while a distal intergenic region of methionine adenosyltransferase 2 like 2 (MAT2L2; Chr22) showed lower levels of methylation in MFO vs. MSO. Descriptive statistics and gene annotation for all DMRs identified in MFO vs. MSO adipose tissue can be found in Supplementary Table 3.

Discussion

Implementing developmental programming through the hen diet has the potential to be a valuable tool to further optimize broiler production. Our objective in this study was to explore the impact of maternal fish oil supplementation on the genome-wide transcriptomic and DNA methylation patterns in the embryonic adipose tissue of broiler chickens.

Enriching the developing embryo in the fatty acids found in fish oil significantly affected the expression of several genes involved in fatty acid activation, a necessary step in the metabolism and storage of fatty acids. Long chain fatty acids are carboxylic acids containing hydrocarbon chains (16-22). To be utilized in cells for various metabolic processes such as β -oxidation, fatty acids are required to be activated. This activation is carried out by one of the 13 acyl-CoA synthetase (ACS) isoforms, including long ACS (ACSL), very-long (ACSVL), fatty acid transport protein (FATP), and ACS bubblegum (ACSBG). Each isoform directs its long-chain fatty acid substrates into specific metabolic pathways, and their functions in different tissues can vary, influencing fatty acid activity and downstream pathways (Grevengoed et al., 2014). Expression of ACSL3, ACSL6, and ACSBG was significantly increased in MFO vs. MSO adipose tissue. Each of these ACSL family members were found to be present in abdominal fat, with particularly high

expression levels in pectoralis (Claire D'Andre et al., 2013). Furthermore, ACSBG2 gene was expressed in abdominal fat, subcutaneous fat, and fatty tissues of breast muscle and pituitary gland. While ACSL1 is the most abundant isoform expressed in chicken adipose tissue, similar to mammals (Bionaz and Looor, 2008; Huang et al., 2015; Tian et al., 2021), the presence of other ACS isoforms in adipose tissues may also contribute to fatty acid utilization in chicken adipose tissue.

Acyl-CoA is a hydrophobic molecule, and its distribution within cells is regulated by fatty acid binding protein (FABP) isoforms to prevent interference with membrane integrity (Füllekrug et al., 2012; Grevenko et al., 2014). In our study, alongside elevated ACS levels, FABP7 exhibited higher level in MFO compared to MSO control. Fatty acid binding protein 7 binds to LC-PUFA, such as ALA, EPA, and DHA, with high affinity to facilitate their uptake, thereby increasing their levels within the cells (Nasrollahzadeh et al., 2008; Elsherbiny et al., 2015; Choi et al., 2021). Collectively these findings suggest that MFO may enhance fatty acid activation and transport for subsequent metabolism and contribute to the local accumulation of EPA and DHA in adipose tissue.

Enriching the embryo with n-3 PUFA through the hen diet decreased the expression of genes related to lipid accumulation, specifically ME1 and PNPLA3 compared to maternal n-6 PUFA. Malic Enzyme 1 (ME1) is a cytosolic enzyme responsible for catalyzing the conversion of malate to pyruvate in the cytosol. During this conversion, ME1 also generates NADPH which is required for fatty acid and cholesterol biosynthesis (Simmen et al., 2020). ME1 is indeed well-known for its pro-adipogenic effects, and its gene expression is susceptible to changes based on dietary fat content (Yang et al., 2009; Al-Dwairi et al., 2012; Huang et al., 2017; Simmen et al., 2023). The expression of ME1 was positively associated with the accumulation of adipose tissue

under a high-fat diet in mice (Yang et al., 2009; Al-Dwairi et al., 2012; Simmen et al., 2023). Silencing ME1 in adipose tissue reduced the expression of the lipogenic gene (FASN), contributing to attenuated adipose tissue deposition and improved insulin sensitivity in mice (Al-Dwairi et al., 2012). Maternal high-fat diet during pregnancy affects the hepatic lipid metabolism of offspring in early life, leading to a significant increase in ME1 expression. This increase results in higher body weight, impaired glucose tolerance, elevated serum cholesterol levels, and hepatic steatosis at weaning (Huang et al., 2017). Furthermore, ME1 expression can be induced by insulin. An elevated carbohydrate intake that stimulates insulin results in an increased expression of ME1 in both liver and adipose tissue (Mariash et al., 1980; Drake and Mucenski, 1985; Simmen et al., 2020).

ME1 expression is associated with fat accumulation in broiler chickens (Hillgartner et al., 1995; Peng et al., 2016). Chickens that exhibited fast growth had a significant amount of subcutaneous and abdominal adipose tissue, which was mainly attributed to their increased feed consumption. This higher feed intake was positively associated with the expression of genes involved in fatty acid synthesis, including FASN and ME1 (Suzuki et al., 2019). Patatin-like phospholipase domain-containing protein 3 (PNPLA3) is highly expressed in both adipose tissue and liver, and it is closely linked to fatty liver disease. When excess PNPLA3 is present on the surface of lipid droplets, it can compete with PNPLA2 (also known as adipose triglyceride lipase, ATGL), for its coactivator (Dong, 2019). This competition occurs because PNPLA3 is structurally closely related to ATGL, sharing 46% sequence homology (Grabner et al., 2021). PNPLA3 on the lipid droplets hinders ATGL activity by interacting with the coactivator, thereby preventing it from binding to ATGL. This interference in coactivation ultimately leads to decreased ATGL activity, resulting in lipid accumulation (Dong, 2019; Grabner et al., 2021). Similarly, in chickens, PNPLA3

is highly expressed in adipose tissues and liver (Claire D'Andre et al., 2013). Interestingly, its expression was significantly higher in fast-growing chickens compared to slow-growing chickens, suggesting that the PNPLA3 gene is involved in fat deposition in chickens.

Expression levels of Twist1 and Crabbp1, both of which are associated with adipocyte inflammation, were significantly increased in adipose tissue of MFO vs. MSO. Twist1 is a transcription factor known for its involvement in the regulation of TNF α signaling and has been found to be highly expressed in both human and mouse adipose tissue. Expression of Twist1 was significantly increased in adipose tissue of MFO vs. MSO. During in vitro differentiation of human preadipocytes, Twist1 levels were shown to increase (Pettersson et al., 2010; Pettersson et al., 2011). Twist1 expression was found to be low in obese subjects but increased after weight loss (Pettersson et al., 2011). Lower Twist1 expression in human white adipose tissue was associated with high TNF α secretion, a proinflammatory cytokine. Additionally, Twist1 levels were negatively related to insulin resistance and adipocyte volume. These findings suggest a complex interplay between Twist1 expression, obesity, inflammation, and metabolic factors in white adipose tissue (Pettersson et al., 2011).

Cellular retinoic acid binding protein 1 (CRABP1) plays a beneficial role in adipocyte biology. Specifically, within adipocytes, CRABP1 is responsible for maintaining mitochondrial homeostasis and regulating the production of adiponectin, which helps to reduce inflammatory responses (Wei et al., 2022). These functions suggest that CRABP1 has a preventive effect on high fat diet-induced adipose tissue inflammation.

Leptin is one the first adipokines that was discovered and is well-established to play a role in regulation of feed intake and other metabolic functions in multiple species. However, the existence of a homolog in chicken is unclear. Nonetheless, the gene encoding the leptin receptor

(LEPR) is well-defined in the chicken genome (Horev et al., 2000; Adachi et al., 2008; Bernardi et al., 2021). Although leptin was not identified, expression of LEPR was higher in MFO vs. MSO. Leptin, an appetite suppressor, regulates food intake and body weight by interacting with leptin receptors in the hypothalamus (Friedman and Halaas, 1998). This hormone is produced by adipocytes and released into the bloodstream, where it affects various tissues, not only the brain but also the liver, muscle, and adipose tissue (Friedman and Halaas, 1998; Kielar et al., 1998). The potential roles of LEPR in different tissues have been discussed. When leptin binds to its receptor in adipose tissue, it activates the Jak/STAT pathway, leading to increased glucose utilization and lipolysis in adipose tissue (Siegrist-Kaiser et al., 1997). Deficiencies in either leptin or the leptin receptor are linked to several metabolic syndromes, especially obesity. High-fat diet feeding led to increased adipose depot weight and a significant decrease in LEPR expression in mouse adipose tissues, indicating reduced leptin sensitivity (Priego et al., 2009). This underscoring the significance of leptin receptor levels within adipose tissue in the context of obesity. Leptin treatment, used as an anti-obesity therapy, enhanced the expression of genes related to acyl-CoA biosynthesis and fatty acid β -oxidation, such as ACSL6 and ACSBG2, in the livers of obese mice (Al-Qeraiwi et al., 2020). This improvement contributed to hepatic lipogenesis and reduced triglyceride accumulation. N-3 PUFA supplementation, such as EPA and DHA, has been shown to improve leptin sensitivity (Pérez-Matute et al., 2007; Hinkle et al., 2013; Huerta et al., 2015; Albracht-Schulte et al., 2018). EPA treatment, for example, has been found to enhance leptin mRNA expression and secretion in 3T3-L1 cells (Pérez-Matute et al., 2005). The increase in basal leptin secretion induced by EPA was strongly associated with elevated glucose utilization. This implies that EPA, similar to insulin, has the potential to enhance leptin production and,

consequently, improve glucose metabolism. Hence, the upregulation of LEPR by MFO may enhance leptin sensitivity, promoting leptin signals and energy expenditure.

The set of DEGs in MFO vs. MSO includes four members of the Iroquois homeobox (IRX) family, each of which showed upregulated expression in MFO. Iroquois homeobox (IRX) genes encode homeodomain transcription factors. Six IRX genes have been identified in chickens and various species (Bosse et al., 2000; Peters et al., 2000; Cavodeassi et al., 2001; Viola and Gonzalez, 2016). This gene family is generally known to regulate gene expression and cell differentiation during early embryonic development (Gomez-Skarmeta et al., 1998; Cardeña-Núñez et al., 2017). Among these, IRX3 has shown particular relevance in adipocyte metabolism. A growing body of evidence from studies in humans and mice suggests that IRX3 plays a crucial role in obesity, particularly through its interaction with the powerful fat mass and obesity-associated (FTO) gene. This interaction allows IRX3 to impact adiposity and adipocyte metabolism (Smemo et al., 2014; Landgraf et al., 2016; Zou et al., 2017; Gholamalizadeh et al., 2019). A study by Landgraf et al. (2016) reported that IRX3 expression was higher in adipocytes isolated from lean children compared to obese children. These findings suggest that increased IRX3 expression may serve as a defense mechanism in protecting body weight. Notably, IRX3 expression is more pronounced in beige and brown adipocytes, where it is known to regulate mitochondrial function through adipose tissue thermogenesis. IRX3 expression has been found to be positively associated with adipocyte browning, a shift from energy-storing white adipocytes to energy-dissipating brown/beige adipocytes. In a study by Zou et al. (2017), elevated IRX3 expression increased mitochondrial mass and triggered the expression of beige markers, including UCP1, PGC1 α , and CIDEA, thereby promoting adipose tissue thermogenesis in both humans and mice. When IRX3 was overexpressed in embryonic adipose tissues of mice, it resulted in increased energy expenditure, reduced lipid

droplets, and lower fat mass. This effect was achieved through the browning program of white adipose tissues, typically induced by cold exposure (Zhang et al., 2021b).

Relatively limited research has been conducted on other IRX genes in relation to adipose tissue. IRX1 expression in human subcutaneous adipose tissue has been found to negatively correlate with BMI (Breitfeld et al., 2020), and was also negatively associated with lipid accumulation and adipogenesis-related genes, such as adiponectin, FABP4, and CEBP α , in preadipocytes of cancer cachexia mouse models (Han et al., 2022). IRX2 expression was observed to be higher in abdominal subcutaneous adipose tissue when compared to gluteal subcutaneous adipose tissue in humans (Karastergiou et al., 2013; Pinnick et al., 2014). While research on IRX4 in adipose tissue is currently lacking, the roles of IRX genes in adipose development in chickens are also unknown.

We investigated DNA methylation in developing adipose tissue in chick embryos and a total of 12 DMRs were identified by MFO vs. MSO (FDR $P < 0.05$). In mice studies, maternal consumption of high fat diet or obesity led to long-lasting effects on the development of adipose tissue in offspring through epigenetic remodeling (Borengasser et al., 2013; Keleher et al., 2018; Zhang et al., 2021a). These effects were driven by DNA methylation pattern of genes involved in adipose tissue differentiation and lipid accumulation. Maternal intake of nutrients, such as n-3 PUFA, can influence the epigenetic patterns in the offspring. Alterations in DNA methylation in response to n-3 PUFA, such as DHA have been reported in human and rodent models (Kulkarni et al., 2011; Lee et al., 2014). For example, perinatal manipulation of n-3 PUFA (α -linolenic acid) intake modulated LC-PUFA metabolism by altering DNA methylation of fatty acid desaturase 2 (Fads2) in maternal and offspring livers in mice (Niculescu et al., 2013). These findings align with the methylation changes induced by maternal n-3 PUFA in our study.

Among 12 DMRs, we identified 7 DMRs corresponding to novel long non-coding RNAs (LncRNAs) that showed hypomethylation in the MFO compared to the control. In addition, 5 of these DMRs were within promoter regions. LncRNAs are defined as RNA molecules that consist of more than 200 nucleotides and do not serve as templates for functional proteins. In general, LncRNAs regulate gene transcription through chromatin modification (remodeling accessible to polymerases), interaction with transcriptional factors, and post-transcriptional control (Squillaro et al., 2020; Statello et al., 2021; Mattick et al., 2023). Furthermore, LncRNAs regulate gene transcription by epigenetic modification, such as DNA methylation and histone acetylation (Zhang et al., 2019). Several studies have demonstrated the role of LncRNAs in adipogenesis and adipose tissue function in humans and mice (Chen et al., 2018; Sun and Lin, 2019; Squillaro et al., 2020). Recent research conducted by Jing et al. (2022) has shed light on the involvement of LncRNAs in the deposition of abdominal fat in young broiler chicks. The study identified several differentially expressed LncRNAs between fat and lean-line broilers, highlighting specific LncRNAs that may play crucial roles in regulating abdominal fat deposition in broilers. Extending upon these insights, the outcomes of the present study may contribute to comprehension of the role of LncRNAs in the context of obesity. Although our data do not support the idea that MFO acts by altering the methylation of DEGs, some of these identified LncRNAs may play a role in regulating gene expression and adipose development.

In addition, we found KIF14 and MAT2L2 were differentially methylated by MFO. Kinesins are a family of microtubule-based motor proteins with pivotal roles in embryonic development (Marszalek and Goldstein, 2000; Konjikusic et al., 2021). They are integral to processes such as proper cell division (mitosis and meiosis), cell shape regulation, and cell migration, all of which are essential for tissue and organ formation (Scholey, 2009; Chen et al.,

2016a). Specific kinesin genes have been linked to distinct embryonic phenotypes, and mutations or dysregulation of kinesins can result in developmental abnormalities or lethality (Carleton et al., 2006; He et al., 2014; Reilly et al., 2019). Kinesin family member 14 (KIF14) is known to have oncogenic functions, primarily due to its atypical overexpression in a variety of cancer types (Abiatari et al., 2009; Singel et al., 2014; Wang et al., 2018). Little is known about the specific function of KIF14 in normal cells. Understanding the specific roles of individual kinesin isoforms in embryonic development is an ongoing area of research. Although our analyses did not provide evidence of a direct role for KIF14 in adipose tissue, potential inhibition of KIF14 related to methylation by MFO may influence chicken embryo development.

Methionine adenosyltransferase (MAT) is an essential enzyme responsible for synthesizing S-adenosylmethionine (SAM), the primary methyl donor in biological processes, from L-methionine and ATP (Cantoni, 1952). Overexpression of its isoforms, MAT2A and MAT2B, has been shown to enhance cell cycle progression in porcine preadipocytes by triggering the phosphorylation of the ERK1/2 (p42/44 MAPK) cascade, a central regulator of cell cycle progression (Zhao et al., 2019). Conversely, when MAT2A and MAT2B are knocked down, cell cycle progression was inhibited. In addition, MAT2B level was lower in subcutaneous adipose tissue in obese pigs compared to lean pigs (Fang et al., 2010). DNA methylation plays a significant role in fetal programming, serving as a critical mechanism for the regulation of gene expression (Van den Veyver, 2002). This regulatory process is highly dependent on the availability of dietary methyl donors, such as methionine, which can exert a substantial influence on gene expression. Consequently, an upregulation of MAT, the enzyme responsible for initiating the conversion of methionine into SAM within the methionine cycle, can enhance the availability of methyl donors (Van den Veyver, 2002; Osorio et al., 2014). This effect has been observed in dairy cows that were

supplemented with methionine during the peripartum period. Such supplementation led to an increase in the gene expression of both MAT isoform (MAT1A) and DNA methyltransferase (Osorio et al., 2014). Consuming a diet abundant in docosahexaenoic acid (DHA) has been demonstrated to elevate MAT activity and enhance MAT expression in liver and lung of rats (Huang et al., 2010). These results suggest that enriching embryonic diets with n-3PUFA in our study may have an impact on methyl donor supply via MAT activity, which, in turn, could also affect methylation and gene expression.

In summary, we have revealed a comprehensive transcriptome and methylome landscape in embryonic adipose tissue, shedding light on genes with the potential to influence adipose development in chickens. This genomic groundwork in chicken embryos not only provides novel mechanistic insights into adipose development but also offers preliminary information to elucidate the epigenetic effects of maternal environmental factors on embryonic development. Further investigations into the sets of differentially expressed genes and differentially methylated loci in response to EPA and DHA uptake are imperative to fully characterize the epigenetic controls influenced by maternal fatty acids.

Appendix

Table 4.1 Summary of DEGs enriched in representative GO terms and KEGG pathways

Term	Count	Up-regulated	Down-regulated
Biological Process			
apoptotic process	26	ROBO2, LEF1, TWIST1, NDNF, TP63, TFAP2A, TFAP2B, WWOX, FZD3, BCL11B, OSR1, OSGIN1, PRLR, SLC25A4, FGFR3, FGFR2, ATF3	RPS6KA2, ORMDL3, TGM2, ANGPT4, EN1, HIC1, TGFB2, BIN1, ELMO1
cell proliferation	19	TFAP2A, TFAP2B, FZD3, BCL11B, OSR1, LEF1, HTR1B, TWIST1, PRLR, NF2L, FGFR3, FGFR2, TP63	VEGFA, TGFB2, RNF139, SP1, RPS6KA2, C6ORF89
embryo development	17	TFAP2A, IRX1, ROBO2, HSPA8, FZD3, OSR1, LEF1, TWIST1, HOXC10, TBX15, TP63, TEAD3, PLS1	MTHFR, EN1, PAX5, TGFB2
cellular lipid metabolic process	13	HEXB, CRABP1, TWIST1, ELOVL7, HACD3, CYP26B1, SCD, LEPR, FGFR3	FGR, ORMDL3, PNPLA3, SOCS4
Cellular Component			
plasma membrane	32	SLC41A2, LY6CLEL, ADK, GPD2, ATP10B, SLC1A3, SLC7A3, KBP, HSPA8, WWOX, FZD3, GMIP, PRLR, GRIN1, PTK7, NF2L, FGFR3, FGFR2, DFNA5	HVCN1, ARHGAP18, NALCN, FGD5, CA2, TGM2, AOC3, SLC2A14, TGFB2, BIN1, C6ORF89, ELMO1, ABCG2
extracellular space	18	HEXB, ASIP, NDNF, NPY, LOC769726	AVBD4, SLC2A14, ANGPT4, AVBD6, SERPIND1, AVBD1, LECT2, CATH2, VEGFA, CATH1, IGLL1, DEFB4A, AVBD7
actin cytoskeleton	9	MYBPC3, PDLIM3, GPD2, MYH11, PLS1, MYLK	ZYX, SNX9
transporter complex	8	CLIC5, KBP, CACNA1B, ATP10B, SCN2A, GRIN1, MYLK	KCNIP1, KCND3

Table 4.1 continued

Term	Count	Up-regulated	Down-regulated
Molecular Function			
nucleic acid binding transcription factor activity	27	SATB1, LEF1, TWIST1, GLIS1, HOXC10, TP63, TEAD3, TFAP2A, IRX1, TFAP2B, IRX2, BCL11B, OSR1, IRX3, IRX4, MYCL, TBX15, LOC430697, HAND2, AHRR, SIM1, ATF3	EN1, PAX5, HIC1, FLI1, SP1
transporter activity	21	CLIC5, SLC41A2, CACNA1B, SLC6A12, SLC1A3, SLC35F1, RYR3, SLC7A3, GRIN1, GJB2, MFSD7, GJB6, KBP, SCN2A, SLC25A4	SLC2A14, KCND3, HVCN1, SLC35E3, NALCN, ABCG2
protein domain specific binding	10	HSPA8, FZD3, TSTD1, LEF1, TWIST1, TP63, YWHAG	SP1, ELMO1, LDB2
cytokine receptor binding	9	PRLR	AVBD4, AVBD6, AVBD1, DEFB4A, AVBD7, VEGFA, JAK1, TGFBR2
KEGG			
Endocytosis	11	HSPA8, IQSEC3, ARPC5L, PARD6G, RAB10L, ACTR3B, FGFR3, FGFR2	BIN1, ARAP3, TGFBR2
Tight junction	8	NF2L, LOC420903, ARPC5L, TUBA4AL, PARD6G, MYH11, ACTR3B	PPP2R2C
Calcium signaling pathway	7	CACNA1B, SLC25A4, FGFR3, FGFR2, RYR3, MYLK, GRIN1	
Fatty acid metabolism	6	SCD, ACSL6, ACSL3, ELOVL7, HACD3, ACSBG2	
PPAR signaling pathway	6	SCD, FABP7, ACSL6, ACSL3, ACSBG2	ME1
Adipocytokine signaling pathway	5	NPY, ACSL6, LEPR, ACSL3, ACSBG2	

Table 4.2 Expression of IRX genes and DEGs associated with lipid metabolism in MFO vs. MSO

ENTREZ ID	Symbol	Product	logFC	FDR
Iroquois homeobox				
374185	IRX1	iroquois homeobox 1, transcript variant X1	2.55	0.016
373975	IRX2	iroquois homeobox 2, transcript variant X1	2.30	0.027
107054273	IRX3	iroquois homeobox 3	1.75	0.041
373974	IRX4	iroquois homeobox 4, transcript variant X1	1.83	0.015
Lipid metabolism				
395706	SCD	stearoyl-CoA desaturase	2.39	0.011
428809	CYP26B1	cytochrome P450 family 26 subfamily B member 1, transcript variant X2	1.42	0.015
396464	NPY	neuropeptide Y	1.31	0.044
374211	CRABP1	cellular retinoic acid binding protein 1	1.17	0.038
374223	LEPR	leptin receptor, transcript variant X1	0.98	0.046
416324	ACSL6	acyl-CoA synthetase long-chain family member 6, transcript variant X2	0.92	0.007
396246	FABP7	fatty acid binding protein 7	0.77	0.050
395491	TWIST1	twist family bHLH transcription factor 1	0.77	0.049
396515	FGFR3	fibroblast growth factor receptor 3	0.71	0.038
431579	ELOVL7	ELOVL fatty acid elongase 7, transcript variant X1	0.51	0.049
424810	ACSL3	acyl-CoA synthetase long chain family member 3, transcript variant X1	0.40	0.028
420090	ACSBG2	acyl-CoA synthetase bubblegum family member 2	0.36	0.019
415539	HACD3	3-hydroxyacyl-CoA dehydratase 3	0.35	0.049
418233	PNPLA3	patatin like phospholipase domain containing 3, transcript variant X1	-0.53	0.031
374189	ME1	malic enzyme 1, transcript variant X2	-0.47	0.049
777583	FGR	FGR proto-oncogene, Src family tyrosine kinase	-0.29	0.032

Table 4.3 Total number of DMRs identified between MSO and MFO adipose tissue based on different levels of significance

Significance	0.005	0.01	0.05	0.1	0.2	0.3
P-value	305	516	1962	3628	6778	9725
FDR	5	8	12	17	87	113

Table 4.4 Top 12 DMRs identified in MFO vs. MSO adipose tissue (FDR $P < 0.05$)

Location	Width	logFC	FDR	CG	Position regarding gene	Distance to TSS	ENSEMBL gene ID	Gene symbol	Description
chr2:95920834-95921311	478	2.38	0.004	20	Distal Intergenic	-16676	ENSGALG00010002030	novel gene	Processed pseudogene
chr8:1475085-1475307	223	1.82	0.043	1	Intron	7707	ENSGALG00010012209	KIF14	kinesin family member 14
chr1:72669443-72669785	343	-2.29	0.001	6	Intron	9028	ENSGALG00010025884	novel gene	LncRNA
chr1:17907027-17907660	634	-2.16	0.004	4	Promoter (≤ 1 kb)	41	ENSGALG00010010622	novel gene	LncRNA
chr1:72678098-72678358	261	-2.08	0.005	1	Promoter (≤ 1 kb)	455	ENSGALG00010025884	novel gene	LncRNA
chr1:141477562-141477785	224	-0.95	0.019	11	Promoter (≤ 1 kb)	-76	ENSGALG00010015088	LOC107050476	uncharacterized
chr3:94947941-94948182	242	-2.22	0.004	1	Promoter (≤ 1 kb)	0	ENSGALG00010004075	novel gene	LncRNA
chr16:2695079-2696807	1729	-2.01	0.009	8	Distal Intergenic	-20730	ENSGALG00010003502	novel gene	LncRNA
chr22:3275322-3275546	225	-0.90	0.043	5	Distal Intergenic	23912	ENSGALG00010019772	MAT2L2	methionine adenosyltransferase 2 like 2
chr36:258980-261726	2747	-1.06	0.005	46	Intron	7358	ENSGALG00010000685	LOC112530906	sodium/glucose cotransporter 2-like
chrW:9099578-9101969	2392	-2.07	0.010	19	Promoter (≤ 1 kb)	0	ENSGALG00010013618	novel gene	LncRNA
chrW:9095860-9097390	1531	-2.10	0.004	9	Promoter (≤ 1 kb)	0	ENSGALG00010013618	novel gene	LncRNA

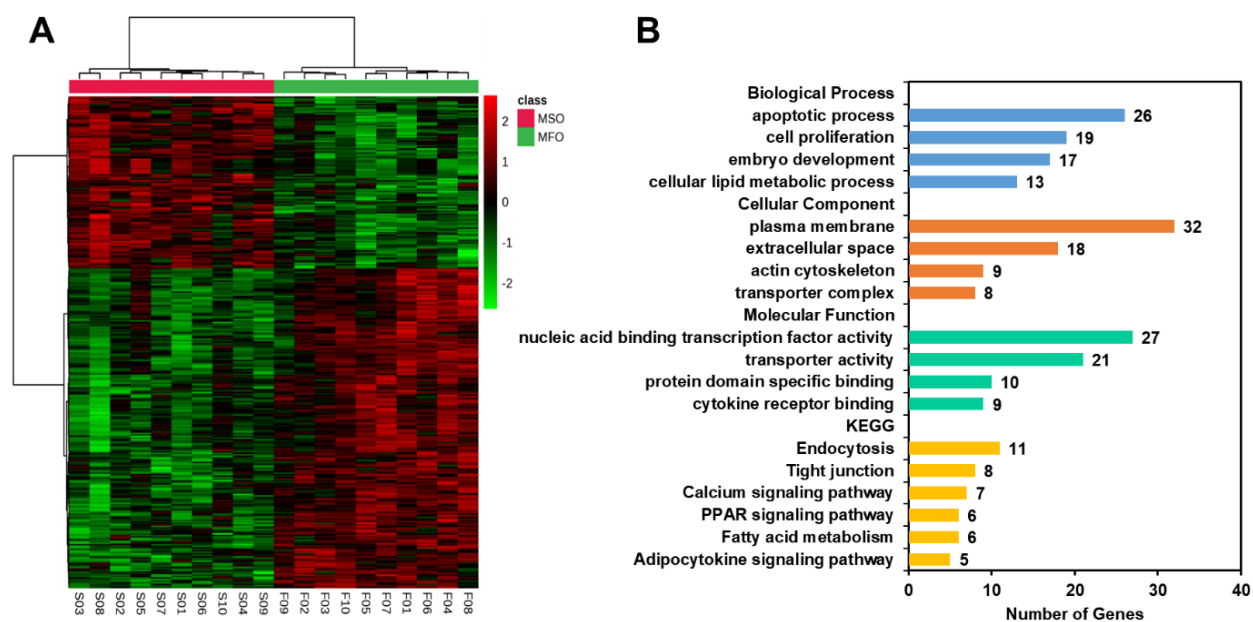


Figure 4.1 Effect of MFO on transcriptome profiling in E16 adipose tissue

(A) Heatmap of 323 DEGs between MSO and MFO (n=10, FDR $P < 0.05$). (B) Bar graph of GO terms and KEGG pathway enrichment analysis.

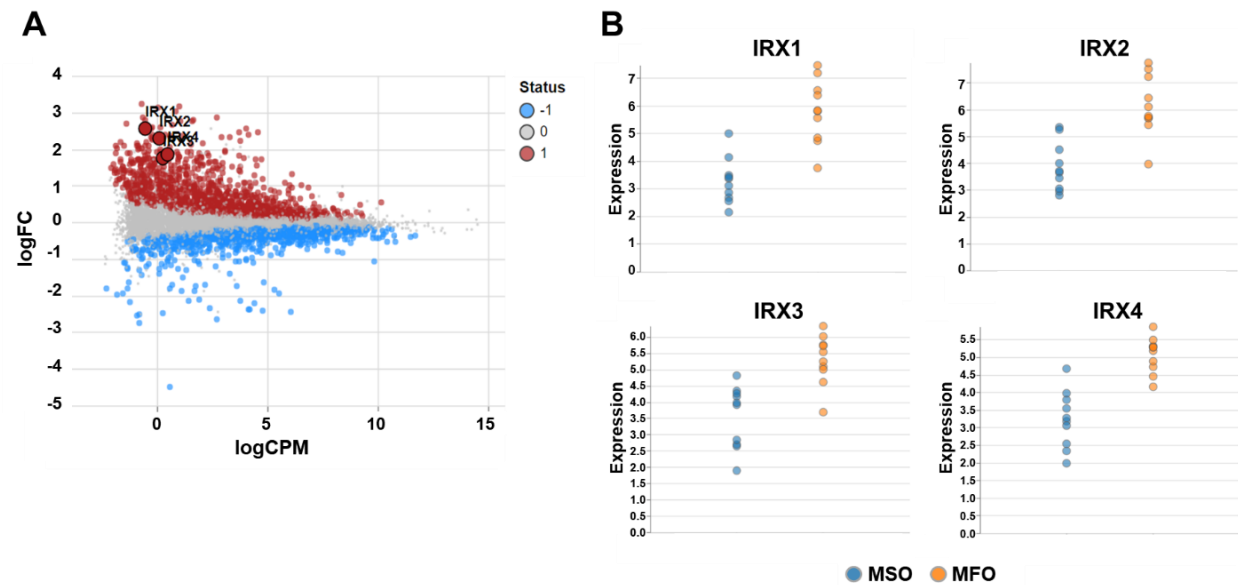


Figure 4.2 Effect of MFO on the expression of Iroquois homeobox (IRX) genes

(A) Scatter plot shows each gene as a point, with the average expression value on the x-axis and the log2 fold-change on the y-axis. Genes are highlighted in red (up-regulated) or blue (down-regulated) (B) Plots illustrate the expression levels of IRX genes for individual sample values (n=10, FDR $P < 0.05$).

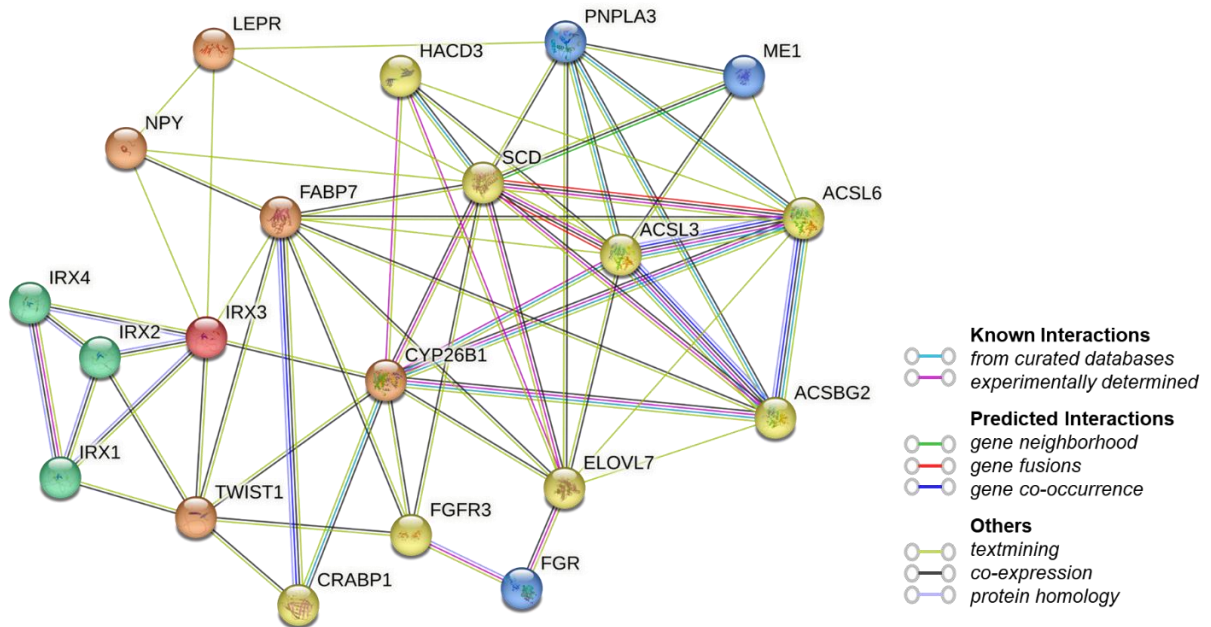


Figure 4.3 Interaction network between protein products of select differentially expressed genes of interest

IRX genes (4) and 16 genes involved in lipid metabolism were input into STRING database (v12.0) using low confidence to identify putative interactions. Line color indicates the type of evidence supporting a pairwise interaction between gene products.

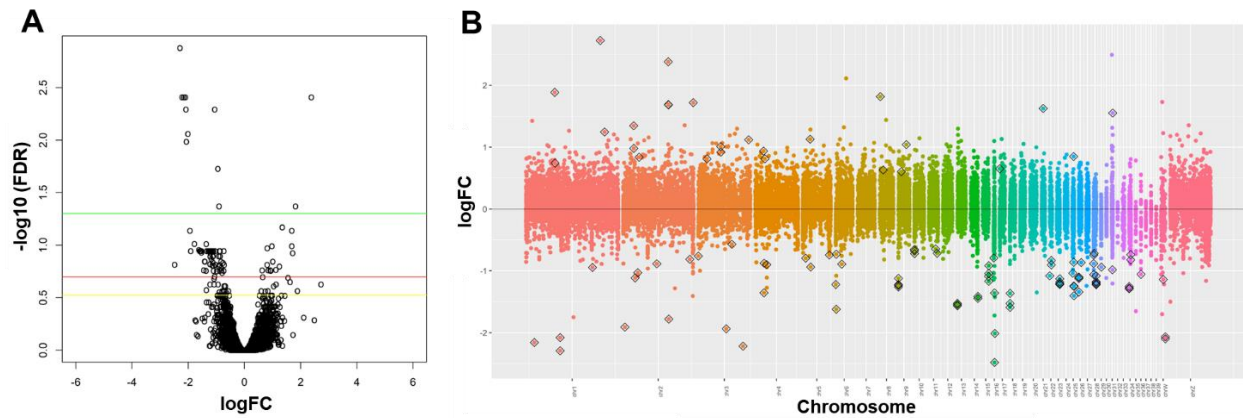


Figure 4.4 Effect of MFO on methylation patterns in E16 adipose tissue

(A) Volcano plot of DMRs shows hypermethylated DMRs on the right and hypomethylated DMRs on the left, with statistically significant genes above three thresholds: FDR $P < 0.05$ (green), 0.2 (red), and 0.3 (yellow). (B) Manhattan plot of $\log_2$ fold change ($\log\text{FC}$) illustrates methylation patterns of DMRs in relation to chromosomal location. The 113 DMRs (FDR $P < 0.3$) are marked with a diamond shape.

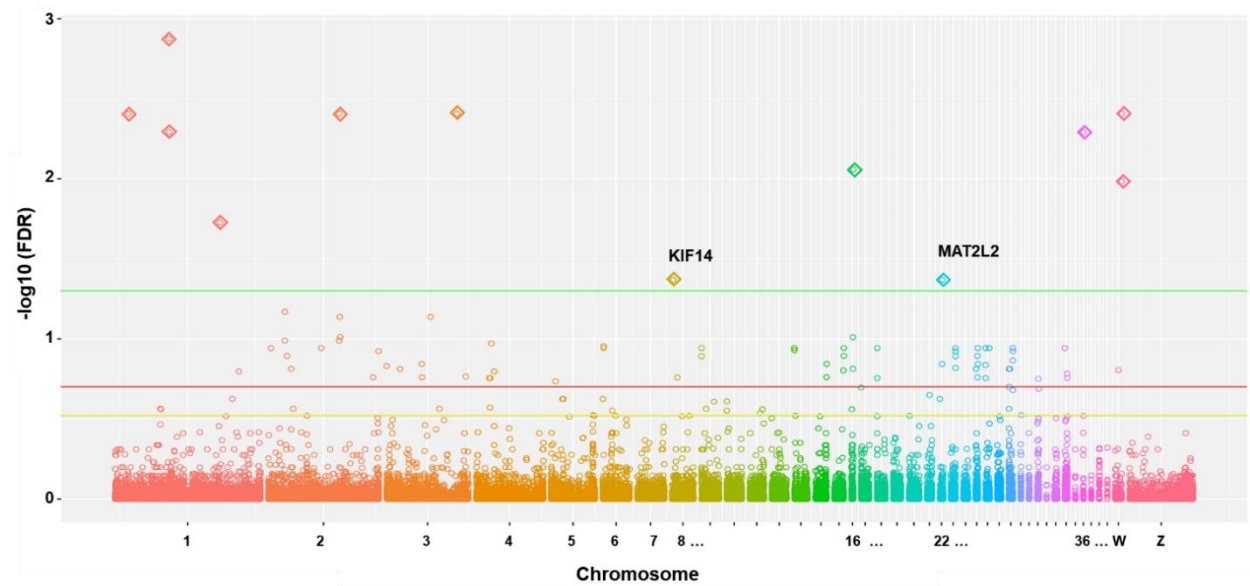


Figure 4.5 Distribution of DMRs identified in MFO vs. MSO adipose tissue

Manhattan plot shows chromosomal location of DMRs mapped on the chicken genome.

Thresholds indicate three significance levels, FDR $P < 0.05$ (green), 0.2 (red), and 0.3 (yellow).

The 12 DMRs (FDR $P < 0.05$) are marked with a diamond shape.

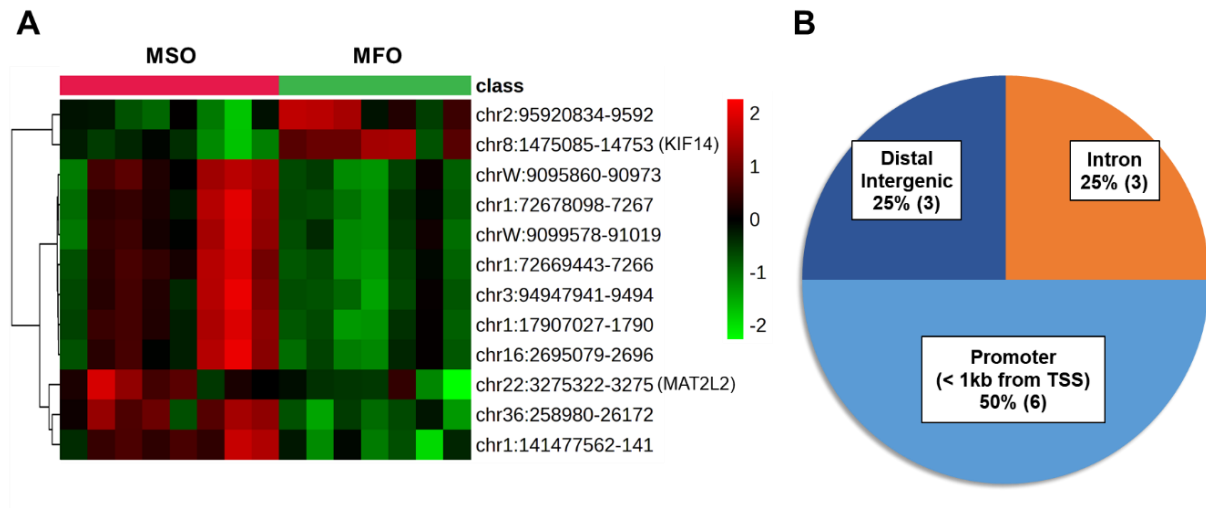


Figure 4.6 Top 12 DMRs identified in MFO vs. MSO adipose tissue (FDR $P < 0.05$)

(A) Heatmap of 12 DMRs (B) Pie chart showing the functional distribution of the 12 DMRs in relation to gene elements, based on the reference chicken genome.

CHAPTER 5 CONCLUSION

Modern broiler chickens waste feed, the most expensive component of production, by partitioning more energy than is necessary into adipose storage rather than muscle growth. This diversion of resources begins shortly after hatch and progresses rapidly, raising the need for strategies that begin to correct this tendency very early in life. Developmental programming is an adaptive physiological process that can be leveraged to limit excess fat deposition and enhance lean growth in broilers. We previously demonstrated that fat accretion in broilers can be reduced by supplementing the broiler hen diet with fish oil, a source of n-3 PUFA, EPA and DHA. Our objective here was to characterize the effects of maternal dietary fish oil (MFO) on adipose development in broiler embryos. This study revealed that MFO significantly enriched yolk and subcutaneous adipose tissue in EPA and DHA. Histological analysis revealed that MFO reduced adipocyte size and increased the proportion of small adipocytes. Preadipocytes isolated from E16 adipose tissue in MFO accumulated significantly less lipid than MSO control. In addition, MFO preadipocytes exhibited a significantly higher mitochondrial activity than MSO preadipocytes, attributed to their increased density, indicating improved oxidative metabolism capacity. Differentially expressed genes (DEGs) associated with adipogenesis, metabolism, and stem cell commitment were identified between the MFO and control groups. Notably, MFO increased the expression of four members of the Iroquois homeobox genes, known to regulate adipogenesis. Additionally, it is worth noting that the type of fatty acids in the hen diet had an impact on DNA methylation patterns, with a total of 12 differentially methylated regions identified between the MSO and MFO groups. Overall, these findings underscore the potential of maternal dietary fish oil to positively influence adipose development and metabolism in broiler embryos, offering a promising avenue to mitigate excess fat deposition and enhance lean growth in broilers from the early stages of life.

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