Effects of Dietary Macronutrient Composition and Exogenous Neuropeptide Y on Adipose Tissue Development in Broiler Chicks

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ABSTRACT (Academic)

The objective of this dissertation research was to investigate the effect of dietary macronutrient composition on neuropeptide Y (NPY)-mediated regulation of adipose tissue physiology in broiler chickens during the early post-hatch period. A high-carbohydrate (HC), high-fat (HF) or high-protein (HP) diet was fed to broiler chicks in all experiments and various facets of physiology were evaluated at day 4 post-hatch, including diet-, fasting-, and neuropeptide Y-induced effects on gene expression, cellular morphology, and lipid metabolism. Experiment 1 was designed to study the effects of diet on molecular changes in different adipose tissue depots (subcutaneous, clavicular and abdominal) after 3 hours of fasting and 1 hour of refeeding. Adipose tissue weights were decreased in chicks that consumed the HP diet, whereas adipocyte diameter was increased in response to the HF diet. There was greater expression of mRNAs encoding fatty acid binding protein 4 (FABP4) and monoglyceride lipase in chicks fed the HC and HF diets than the HP diet in all three adipose tissue depots. Fasting increased plasma non-esterified fatty acid concentrations in chicks fed the HC and HP diets. Results suggest that the heavier fat depots and larger adipocytes in chicks fed the HF diet are explained by greater rates of hypertrophy, whereas the HP diet led to a decrease in adipose tissue deposition, likely as a result of decreased rates of adipogenesis. Experiments 2 and 3 were designed to investigate how dietary macronutrient composition affects the effect of centrally or peripherally administered NPY, respectively, on lipid metabolism-associated factor mRNAs in adipose tissue. In experiment 2, vehicle or 0.2 nmol of NPY was injected intracerebroventricularly (ICV) and

abdominal and subcutaneous fat samples were collected at 1 hour post-injection. In the subcutaneous fat, ICV NPY injection decreased peroxisome proliferator-activated receptor γ (PPARγ) and sterol regulatory element-binding transcription factor 1 (SREBP1) mRNAs in chicks fed the HF diet, whereas there was an increase in SREBP1 expression in chicks fed the HF diet after NPY injection. Expression of PPARγ and FABP4 mRNAs increased in the abdominal fat of HF diet-fed chicks after NPY injection. Thus, HF diet consumption may have enhanced the sensitivity of chick adipose tissue to the effect of centrally-injected NPY on gene expression of adipogenesis-associated factors. In experiment 3, vehicle, 60, or 120 µg/kg BW of NPY was injected intraperitoneally (IP), and subcutaneous, clavicular, and abdominal fat was collected at 1 and 3 hours post-injection. Food intake and plasma NEFA concentrations were not different among chicks fed the HC, HF or HP diet after IP NPY injection, indicating that the effects of NPY on adipogenesis were independent of secondary effects due to altered energy intake. In response to the lower dose of NPY, the expression of NPY receptor sub-type 2 mRNA was increased at 1 hour post-injection in the subcutaneous fat of chicks fed the HP diet, whereas there was less 1-acylglycerol-3-phosphate O-acyltransferase 2 mRNA in the subcutaneous fat of chicks fed the HC diet. The higher dose of NPY was associated with greater AGPAT2 mRNA in the clavicular fat of chicks that consumed the HP diet and less CCAAT/enhancer-binding protein alpha in the abdominal fat of chicks that were provided the HF diet. However, there was also a decrease in the expression of some of these factors, although mechanisms are unclear. In conclusion, dietary macronutrient composition influenced the response of adipose tissue to the adipogenic effects of NPY and metabolic effects of short-term fasting and refeeding during the first week post-hatch. Collectively, this research may provide insights on understanding NPY's effects on the development of adipose tissue during the early life period and mechanisms

underlying diet-dependent and depot-dependent differences in adipose tissue physiology across species.

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Neuropetide Y (NPY) is a 36 amino-acid peptide that increases hunger and fat deposition. The objective of this dissertation research was to elucidate how dietary fat/protein affect NPY's effect on fat tissue physiology in broiler chicks during the early post-hatch period. Three diets that were formulated to be high-carbohydrate (HC), high-fat (HF) or high-protein (HP) were fed to broiler chicks from day of hatch to day 4 post-hatch. In experiment 1, chicks were fasted for 3 hours and refed for 1 hour after 3 hours of fasting. Adipose tissue weight was decreased in chicks fed the HP diet and the diameter of fat cells was greater in chicks that consumed the HF diet. In the adipose tissue of chicks fed the HP diet there was reduced gene expression of factors associated with lipid synthesis and fat cell development. Fasting increased plasma free fatty acid concentrations in chicks fed the HC and HP diets. Results suggest that HP diet-induced decreases in fat deposition might be explained by a decrease in rates of fat cell development/maturation. However, chicks fed the HF diet had more fat deposition and larger fat cells, likely as a result of hypertrophy (growth in cell size). Experiment 2 was designed to investigate how NPY administration in the central nervous system affects adipose tissue physiology after feeding the three diets. Subcutaneous, clavicular and abdominal fat samples were collected at 1 hour post-injection. The injection of NPY increased the gene expression of factors associated with fat cell development and maturation in the abdominal fat of chicks fed the HF diet. Thus, HF diet feeding might have sensitized chicks to the effect of centrally-injected NPY on adipose tissue to deposit more fat and increase the number/size of fat cells. In experiment 3, NPY was injected into the peritoneum of chicks fed the HC, HF and HP diets. Although NPY injection increased the gene expression of factors involved in lipid synthesis and fat cell development/maturation, there was also a decrease in the expression of these factors, yet the mechanisms are unknown.

Food intake and plasma free fatty acid concentrations were not affected in response to NPY injection at 1 or 3 hours post-injection, indicating that the effect of NPY on fat cell development and lipid synthesis is independent of secondary effects due to altered energy intake. Overall, this research may provide insights on understanding the effect of NPY on fat cell development and has implications for improving animal production efficiency by increasing feed conversion into muscle instead of fat and minimizing excess fat deposition during certain stages of growth.

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Chapter 1: Introduction

With intensive selection for traits that maximize meat production, the growth rates of broiler chickens have increased by 50% over the past 30 years (slaughter live weight, 2018: 6.3 lb; 1985: 4.2 lb) (1), which has been accompanied by increased body fat deposition, more skeletal disorders, and a greater incidence of metabolic diseases and mortality (2, 3). Increased fat accumulation in broiler chickens raises economical concerns (4, 5), because excessive fat can hinder processing and may also result in rejection of the meat product by consumers, and reduce feed efficiency and carcass yield (6). In the previous studies evaluating adipose deposition in chickens, the deposition of subcutaneous fat was not reported and the abdominal fat was the focus (7). Abdominal fat pad weight as a percentage of slaughter body weight of broiler chickens (different genetic strains such as Ross PM3 and Ross × Ross 308) on day 42 has been relatively unchanged during the past decade (2008: 1.52 -2.20%; 2013: 1.55-2.22 %; 2018: 1.81-2.22%) (8-13).

Because intense selection has also resulted in a much shorter time period for broiler chickens to reach market weight, the early post-hatch period is a much greater proportion of a market broiler's life. In addition, chicks are without access to feed and water for about 48-72 hours after hatching under practical conditions, resulting in a shortage of energy (14). When feed is first provided to chicks, they shift their metabolic dependence from endogenous lipid-rich yolk to exogenous carbohydrate and protein-rich feed (15). Concurrently, intense morphological and functional development also occurs in the digestive tract (16). Hence, research during the early post-hatch period, including consequences of food deprivation and compensatory effects associated with refeeding, and the mechanisms that regulate nutrient partitioning to adipose tissue may lead to strategies that further increase production efficiency.

Additionally, chickens serve as a valuable research model for human diseases. For instance, chickens exhibit hyperglycemia (up to 200 mg/dL in the fasting state) and resistance to exogenous insulin, thereby mimicking the early stage of type 2 diabetes in humans (17, 18). Unlike rodents or pigs, *de novo* lipid synthesis occurs mainly in the liver instead of the adipose tissue, which is similar to humans (19, 20), thus the chicken might be a more representative model to use for studying the factors that regulate lipogenesis and lipid trafficking. Because of the intense selection for growth-related traits, the compulsive feeding behavior and propensity to become obese make the broiler an attractive model for studying obesity and metabolic syndrome.

The literature review in this dissertation will focus on adipogenesis, lipid synthesis and breakdown, and factors that affect these activities in adipose tissue in chickens, with an emphasis on dietary factors. As will be discussed in Chapter 2, dietary macronutrient composition and neuropeptide Y (NPY) are two factors that are the focus of this dissertation research. The main objective was to investigate the effect of dietary macronutrient composition on the regulation of NPY-mediated roles in fat deposition during the early post-hatch period. In mammals, the molecular and cellular mechanisms underlying adipose tissue development have been well-studied. The development of adipose tissue is a consequence of both multiplication of new cells that can be induced to differentiate into adipocytes (hyperplasia) and increased accumulation of lipids in adipocytes (hypertrophy). However, there is still a paucity of knowledge on adipose tissue development in avian species. Thus, research from this dissertation may contribute to the understanding on adipose tissue physiology and provide insight on strategies to formulate diets that maximize conversion into muscle rather than fat. Since chickens are also a valuable model for studies of human obesity (21), the results from this dissertation may also contribute to the understanding of obesity and metabolic disorders in humans.

Chapter 2: Literature review

Factors affecting adipose tissue development in chickens: A review

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Adipogenesis and adipose tissue expansion

Adipogenesis overview. Mesenchymal stem cells are multipotent stromal cells with the capacity to differentiate into a variety of cell types, such as myoblasts, osteoblasts, chondrocytes and adipocytes (22). Thus, adipocytes are derived from connective tissues, and once a mesenchymal stem cell is committed to the preadipocyte pathway, maturation and growth is completed by synchronized actions of various transcription factors, discussed in later sections of this review. Preadipocytes can continue to proliferate but once committed to becoming an adipocyte, terminal differentiation is characterized by the accumulation of lipids (i.e., triacylglycerol; TAG). Tissue development is associated with collagen synthesis and angiogenesis to provide structural support, nutrients, and oxygen to the cells. Multiple lipid droplets (LDs) that form inside the differentiating cell (i.e., multilocular) start to coalesce into a single droplet (i.e., unilocular) during cellular maturation.

Hyperplasia and hypertrophy. The cellular development of adipose tissue thus includes both an increase in cell size (hypertrophy) and an increase in cell number (hyperplasia). Both adipocyte number and size increase with age, which show a positive correlation with body mass and fat pad weight in chickens (23). Thus, hyperplasia and hypertrophy of adipocytes contribute to fat accumulation in chickens, but with different magnitudes of contribution to the volume and weight of the distinct adipose tissue depots.

During chicken embryonic adipose tissue development, preadipocyte hyperplasia dominates, followed by hypertrophy to establish immature adipocytes that are receptive to lipid deposition (24). In Leghorns and broilers, hyperplasia is induced in neck and leg fat pads between embryonic day 12 and day 14, followed by slowing rates of hyperplasia as embryos reach day 18 (25). Most of the adipocytes are unilocular once the embryo reaches day 14 of incubation, with multilocular cells almost undetectable at this stage, indicating that chicken adipocytes undergo rapid maturation during embryonic development (25).

Lipid droplet composition. Lipid droplets, which represent the majority of the volume of an adipocyte, are fat-storing organelles composed of a hydrophobic core of TAG and cholesterol esters. LDs can be formed in nearly all cells. In the LD core, the primary neutral lipids are sterol esters and TAG, with the primary form being TAG in LDs of adipocytes. The hydrophobic core is separated from the aqueous cytosol by a phospholipid monolayer that contains various proteins (26), including enzymes involved in lipid synthesis, lipases, and membrane-trafficking and structural proteins (27).

De novo fatty acid synthesis. The synthesis of TAGs in adipocytes requires a steady supply of free fatty acids. The liver and adipose tissue are the two main sites of de novo fatty acid synthesis in higher vertebrates (Figure 2-1). In avian species, the liver is the main site of de novo lipogenesis, where glucose is catabolized to acetyl-CoA, which is then converted into fatty acids and cholesterol. In the liver, cholesterol and TAG are incorporated into very low density lipoproteins (VLDLs) to be transported to other tissues via the circulation.

TAG synthesis in adipose tissue. TAG synthesis requires both nonesterified fatty acids (NEFAs) and glycerol 3-phosphate. The majority of fatty acids in the adipose tissue are taken up as fatty acids from TAG in plasma lipoproteins (e.g., VLDL) that are synthesized and packaged by the

liver. Fatty acids may also be acquired from dietary fat delivered to the circulation from the intestine as chylomicrons (28). Thus, the fatty acid composition of the diet in non-ruminant species has a direct bearing on the fatty acid profile of lipids stored in adipose tissue. In chickens, long chain fatty acids are assembled into packages called "portomicrons" that enter directly into the portal blood from the small intestine, with the lipid composition and size of lipoproteins closely resembling that of rat chylomicrons (29, 30). The hydrolysis of TAG in VLDLs is catalyzed by lipoprotein lipase (LPL) on endothelial cells lining the adipose tissue, a rate-limiting step in fat accretion in chickens (31). Therefore, in chickens, fatty acids for TAG synthesis and adipose tissue expansion are acquired from plasma VLDLs and portomicrons.

The glycerol generated from the hydrolysis of TAG cannot be re-utilized to esterify fatty acids due to the lack of glycerol kinase in the adipose tissue. During fasting, adipose tissue obtains the glycerol 3-phosphate required for TAG synthesis from pyruvate via gluconeogenesis or glucose via glycolysis (32-35). The process of adipocyte hypertrophy involves the continued accumulation of TAG inside the LD and "stretching" of the cell.

Acyl-CoA is synthesized from fatty acids and CoA. For glycerolipid biosynthesis, the acylation of glycerol 3-phosphate is the first and committed step. The reaction to produce 1-acyl-sn-glycerol 3-phosphate (lysophosphatidate (LPA)) is catalyzed by acyl-CoA:glycerol-sn-3-phosphate acyltransferase (GPAT) (36). Phosphatidate is synthesized by the acylation of LPA in a reaction catalyzed by acyl-CoA:1-acylglycerol-sn-3-phosphate acyltransferase (AGPAT). The phosphatidate formation catalyzed by AGPAT marks a central branch point in lipid biosynthetic pathways (36). One of the possible routes for phosphatidate is dephosphorylation by phosphatidic acid phosphatase to produce diacylglycerol (DAG) (37). As the final step in TAG

synthesis, diglyceride acyltransferase (DGAT) catalyzes the formation of TAG from DAG and fatty acyl-CoA.

Lipolysis. When animals are not in an energy-deficient state, NEFAs originate from ingested TAG, whereas plasma NEFAs come almost entirely from hydrolysis of TAG in the adipose tissue during the fasting state (38). Net accretion of fat is the balance between intestinally-absorbed fat from the diet, fatty acid and TAG synthesis, and fat breakdown. Lipolysis is the mobilization of NEFAs from stored TAG, which occurs sequentially through a series of hydrolysis reactions (Figure 2-1). Adipose triglyceride lipase (ATGL), also known as desnutrin, is a rate-limiting enzyme that catalyzes hydrolysis of the first ester bond of a TAG inside the LD to release a fatty acid and DAG (39, 40). The action of ATGL is followed by hormone-sensitive lipase (HSL) and monoacylglycerol lipase (MGL) (41). HSL hydrolyzes DAGs and MGL liberates the final fatty acid from the glycerol backbone. In mammalian cells, activation of HSL is achieved by phosphorylation, which occurs via cyclic AMP dependent protein kinase (42). Members of the PAT family of proteins, originally named for perilipin, adipose differentiation-related protein and tail interacting protein 47, serve important functions in regulating lipolysis (43, 44). It appears that there is no HSL orthologue in the chicken genome (45).

NEFAs are released into the circulation and transported to other tissues, such as skeletal and cardiac muscles and the liver, for β-oxidation to generate ATP. The major proteins that are involved in fatty acid transport in mammals are CD36/FAT (fatty acid translocase), fatty acid transport proteins (FATPs) and fatty acid binding proteins (FABPs), which have also been described in avian adipose tissues (46-49). Glycerol is transported to the liver, where it feeds into glycolysis to provide energy or can be used in gluconeogenesis.

Metabolic differences between adipose tissue depots. The physiology of different fat depots in mammals has been intensely studied, and has led to the consensus that different fat depots from various body regions are metabolically distinct. Subcutaneous fat has been reported to act as a buffer for the daily incorporation of dietary fat, preventing other tissues from receiving excessive lipids that can lead to lipotoxicity (50). However, visceral fat (body fat stored within the abdominal cavity) is related to metabolic disorders (51). In avian species, there is a paucity of knowledge regarding the metabolic characteristics of different fat depots. Our group reported that the average size of adipocytes was similar between abdominal and subcutaneous fat in chickens, while gene expression profiles of adipogenesis-associated factors differed (52). From an agricultural perspective, subcutaneous and abdominal fat can be regarded as waste in the slaughterhouse, while intramuscular fat may be regarded as favorable in relation to meat quality. The growth pattern of fat depots can be altered by dietary fatty acid composition. For instance, consumption of a diet containing more unsaturated than saturated fatty acids (SFAs) was associated with a 9 and 30 % reduction in subcutaneous and abdominal fat, respectively, at 42 days post-hatch in broilers (53).

Chickens as a model to study adipose development. Chickens have been widely used for developmental and immunology studies because of several advantages as a model. Development occurs inside of the egg, is independent of maternal influence and manipulations, and viewing can be accomplished easily through introducing holes into the eggshell. Chickens are also a model for studies of adipose tissue biology and human metabolic disorders. Commercial broiler chickens have been intensely selected for growth rate, meat yield, and feed conversion efficiency over the past 70 years. This increase in growth has been accompanied by an increase in voluntary food intake, increased fat deposition in the body, and higher incidence of metabolic diseases (2,

54). Similar to humans, but unlike pigs and rodents, chickens use the liver rather than adipose tissue as the main site of *de novo* lipid synthesis (19, 55). Because of natural hyperglycemia (up to 200 mg/dL in fasting state) and a relative resistance to the effects of insulin in insulin-dependent tissues such as skeletal muscle and adipose, chickens mimic the early stages of type 2 diabetes in humans (20, 56). Therefore, understanding the mechanisms of adipose tissue development in chickens can benefit the poultry industry and also provide insight into biomedical research.

Transcription factors involved in adipogenesis

Various signaling pathways are involved in the commitment of mesenchymal stem cells towards an adipogenic lineage, including the β-catenin-dependent Wnt and Hedgehog signaling pathways (57). Wnt signaling family members suppress the early stages of adipogenesis, maintaining preadipocytes in an undifferentiated state via inhibition of peroxisome proliferator-activated receptor γ (PPARγ) and CCAAT/enhancer binding protein-α (C/EBPα) (57). Similar to Wnt, Hedgehog signals also act to repress adipogenesis through induction of anti-adipogenic transcription factors such as GATA binding protein 2 (GATA2). The differentiation of preadipocytes and regulation of lipid synthesis and metabolism are processes mediated by various transcription factors (Figure 2-2).

Early transcriptional regulators C/EBP$_\beta$ and C/EBP$_\delta$ are expressed transiently, followed by the induction of key transcription factors PPARγ and C/EBPα that coordinate the transcriptional regulation of a variety of adipocyte-associated genes (58). PPARγ is considered to be the "master" regulator of adipogenesis. Its expression is up-regulated very early during differentiation (59). Both in vitro and in vivo studies showed that the formation of the adipocyte cannot be completed without PPARγ (60, 61). Terminal differentiation is promoted by the activation of PPARγ, which

is achieved through the induction of a variety of differentiation-dependent genes that are crucial for TAG uptake and storage, such as FABP4, LPL and others. The importance of PPARγ in avians is also confirmed by a report that the suppression of PPARγ mRNA by small-interfering RNA inhibited differentiation and promoted proliferation of chicken preadipocytes (62).

In mammals, C/EBPα is induced later than PPARγ, and is a critical regulator of adipocyte differentiation; expression of C/EBPα is positively regulated by PPARγ (63). Although PPARγ is able to drive cells towards the adipogenic program in the absence of C/EBPα, C/EBPα is incapable of inducing adipogensis without PPARγ (64).

The zinc finger protein 423 (ZFP423) was identified as a regulator of preadipocyte cell determination and is more abundant in preadipose than non-preadipose fibroblasts (65). Inhibition of ZFP423 in a murine preadipocyte cell line (3T3-L1) blocks PPARγ expression and adipogenic differentiation, whereas ectopic expression of ZFP423 in non-adipogenic fibroblasts (NIH 3T3) induces expression of PPARγ in undifferentiated cells and enhances adipogenesis once cells have been induced to differentiate (65).

Krüppel-like transcription factors (KLFs) and sterol regulatory element binding proteins (SREBPs) are other transcription factors that facilitate adipocyte maturation. KLF5 is activated by C/EBP$_{\beta}$ and C/EBP$_{\delta}$, and acting jointly with these C/EBPs, contributes to the induction of PPARγ (66). Other members of the KLF family, such as KLF6 and KLF15, promote adipogenesis (67, 68); however, there are also KLF members that are reported to be anti-adipogenic. For instance, KLF2 binding suppresses transcription from the PPARγ promoter (69, 70) and KLF7 enhances chicken chicken preadipocyte proliferation and suppresses differentiation (71).

The GATA binding protein 2 (GATA2) is reported to be a preadipocyte-specific factor that promotes preadipocyte activity (72) as its overexpression inhibited chicken preadipocyte differentiation (73). Preadipocyte factor-1 (Pref-1) is a transmembrane protein that is highly expressed in preadipocytes and also acts to inhibit adipocyte differentiation, possibly through suppression of C/EBPα and PPARγ expression (74).

SREBPs are basic helix-loop-helix-leucine zipper transcription factors that play important roles in regulating expression of genes involved in the biosynthesis of cholesterol and fatty acids (75). Most of the lipogenic enzymes, such as acetyl-CoA carboxylase$_{\alpha}$ (ACC$_{\alpha}$) and fatty acid synthase (FAS) are regulated by SREBP (76).

Fatty acid-binding protein 4 (FABP4), an adipocyte differentiation marker, plays an important role in fatty acid transportation and metabolism, and its expression is also regulated by PPARγ (77, 78). The expression of FABP4 is increased dramatically as differentiation progresses (79), and contributes to hypertrophy by mediating the sequestration of fatty acids for TAG synthesis (80).

Hormones that regulate adipogenesis

In mammalian models, a glucocorticoid (dexamethasone), a phosphodiesterase inhibitor (3-isobutyl-l-methylxanthine (IBMX)) and insulin induce adipocyte differentiation in vitro, and are often included together in what is referred to as an "adipose differentiation cocktail" (81-83). However, the mixture of these three factors may not be sufficient to promote chicken preadipocyte differentiation (84). Dexamethasone is a strong inducer during the early stages of adipogenesis and directly enhances transcription of master regulators, such as PPARγ, C/EBP$_{\beta}$ and C/EBP$_{\delta}$ (85, 86). Porcine insulin increases the activity of known enzyme markers for

chicken preadipocyte differentiation, such as glycerol-3-phosphate dehydrogenase and citrate lyase (87).

In recent years, appetite-regulating peptides have emerged as factors that not only regulate food intake via the hypothalamus, but also act on the adipose tissue to regulate energy storage and expenditure. Neuropeptide Y (NPY) is a potent orexigenic peptide in both mammalian (88) and non-mammalian species (89). In addition to appetite regulation, NPY plays other important roles in the body including regulation of energy homeostasis (90). Activation of NPY receptor 2 (NPYR2) enhances adipocyte proliferation and differentiation, and treatment with NPY can lead to obesity in mammalian species (91). Consistent with this, NPYR2 knockout mice had reduced body weight gain and less adiposity (92). Although NPY receptors 1 (NPYR1) and 5 (NPYR5) are implicated in the effect of NPY on fat accumulation, there are fewer supporting studies compared to those focused on NPYR2 (93, 94). NPY also promotes adipogenesis in chicken adipose cells in vitro, which may involve slightly different mechanisms from those identified in mammals (95, 96).

Dietary factors that affect adipogenesis and adipose tissue expansion in chickens

The remainder of this review will focus on dietary factors affecting adipogenesis in chickens (Table 2-1). While there was a review on the relationship of diet to adipose tissue development in chickens (97), this will focus on the molecular and cellular mechanisms underlying effects of dietary factors on the adipose tissue.

Lipid source. At hatch, the chick transitions from the nourishment of a lipid-rich yolk to a carbohydrate- and protein-based diet, during which time there is dramatic morphological and functional development of the gastrointestinal tract (98, 99). When chicks were fed a high fat

diet (9% vs 4.5% crude fat; diets supplemented with soybean oil) or high protein diet (28% vs 18% crude protein) from hatch, carcass fat percentage did not differ at 7 days of age (100). Absorption of fatty acids from dietary fat is considered to be efficient at hatch and because consumption of a high fat diet was not accompanied by increased body fat deposition as seen in older chickens, it was suggested that mechanisms to enhance deposition of energy as adipose tissue are not yet in place during the first week post-hatch (100). This is in contrast to older female chickens (12 to 49 d of age) where an increase in dietary fat (tallow or olive oil) from 6% to 10% was associated with increased abdominal fat (101). In general, as the animal matures, the amount of body fat increases while muscle decreases, and the deposition of adipose tissue is influenced by the energy to protein ratio in the diet (102, 103).

In addition to the energy density and crude fat percentage of the diet, the source of fat also affects body fat deposition. Chickens fed diets containing sunflower oil (predominately composed of unsaturated fatty acids) as the major source of supplemental fat had less abdominal fat than those fed diets supplemented with tallow or lard (greater proportion of saturated fatty acids) (104). Inclusion of sunflower oil in the diet was associated with reduced plasma TAGs and FAS activity in the liver and enhanced carnitine palmitoyltransferase I and L-3-hydroxyacyl-CoA dehydrogenase activities in the heart, as compared to the physiology of birds that ate diets containing tallow (105), suggestive of enhanced rates of β oxidation in the heart and reduced lipogenesis in the liver. These results indicate that energy-demanding tissues such as skeletal muscle enhance their uptake of fat as an energy source from sunflower oil, preventing deposition as fat (105).

Fatty acids. The effects of dietary fat source on adipose tissue physiology may be due in part to the effects of individual fatty acids on metabolism. Fatty acids are ubiquitous biological

molecules that besides providing energy and constituting cellular membranes, can also function as mediators of adipocyte differentiation and metabolism in mammals (106, 107). Fatty acids are important for the induction of chicken adipocyte differentiation, the effect associated with an increase in PPARγ gene expression (108). Fatty acids regulate gene transcription by serving as regulatory ligands for specific transcription factors or by being involved in intracellular metabolism, such as by serving as substrates for oxidation, TAG synthesis or as precursors for other biological molecules (109). Fatty acids serve as PPAR ligands (110, 111), which may partly explain why fatty acids and their derivatives have hormone-like effects. Thus, fatty acids can affect preadipocyte proliferation and differentiation by regulating transcription of specific genes and also by serving as substrate for the accelerated TAG synthesis that occurs during adipocyte maturation. The expression of C/EBPs, PPARs and other adipose-specific genes is regulated by the presence of fatty acids during the early phase of adipocyte development in both mammals (112) and in birds (84).

Because the liver is the main site of fatty acid synthesis in chickens, it has been suggested that in a cell culture system, additional fatty acid supplementation may be required to promote differentiation of chicken adipocytes (84). Oleic acid was shown to be an essential source of exogenous fatty acids for chicken adipocyte differentiation in vitro when fetal bovine serum was used instead of chicken serum to supplement the media (84). In one study, oleate alone (ie., absence of differentiation cocktail) was able to stimulate lipid droplet formation in chicken preadipocytes (72). The same group also reported that oleate has the capacity to promote trans-diffferentiation of chicken fibroblasts into adipocyte-like cells (113). The adipogenesis-inducing effect of oleic acid in laying hen adipocytes was explained by the corresponding increase in gene expression of FABP4 and C/EBP$_\beta$ (114, 115).

Conjugated linoleic acid (CLA) is a mixture of mainly cis-9, trans-11 and trans-10, cis-12 fatty acid isomers. Many in vivo and in vitro studies showed that dietary CLA mediates cell growth, nutrient utilization and storage, and lipid metabolism in rodents and pigs, and that when fed to animals it partitions energy from fat to skeletal muscle growth (116, 117). Total body fat was reduced in chickens fed 2 or 3 % dietary CLA from 3 to 6 weeks of age (118). In rats, the CLA-induced decrease in body fat mass was because of a decrease in adipocyte size, rather than adipocyte number (119). Dietary CLA also increases SFA and decreases monounsaturated fatty acid (MUFA) content in the abdominal fat of chickens, which most likely results from inhibiting the Δ-9 desaturase enzyme system that is responsible for SFA desaturation, converting SFA into MUFA (120).

When supplemented in the diet, polyunsaturated fatty acids (PUFAs) decrease abdominal fat (101), fat in other depots (121) and total body fat (122), compared to saturated or monounsaturated fats. This may be explained by results from studies suggesting that PUFA suppress fat synthesis (105, 123, 124), and increase fatty acid oxidation (125-127), in both mammals and birds. The majority of metabolic effects of fatty acids consumed are controlled at the level of gene transcription regulation, either indirectly or directly by nuclear hormone receptors such as PPARs (128, 129). PUFA and CLA isomers act as ligands for PPARs (117, 130), and dietary CLA is associated with decreases in PPARγ mRNA abundance in chicken abdominal fat (131). However, a combination of a variety of long-chain fatty acids induced chick adipocyte differentiation by upregulating PPARγ mRNA and protein expression (108). This may indicate that fatty acids with different structures may act on adipogenesis through different mechanisms.

Compared to long-chain fatty acids (LCFAs), medium-chain fatty acids (MCFAs) decrease fat deposition in mammals due to faster metabolism and reduced storage in adipocytes (132, 133). In chickens, MCFA may be more advantageous in reducing abdominal and intermuscular fat than LCFA (134). However, the specific mechanisms are not fully understood. Thus, both the degree of saturation and the chain length are associated with different metabolic functions of fatty acids in chickens.

Dietary carbohydrates. The ACC is considered to be a rate-limiting step in the fatty acid synthesis pathway, catalyzing the ATP-dependent carboxylation of acetyl-CoA to malonyl-CoA to donate carbon atoms for long-chain fatty acid synthesis (135). The activity of ACC also controls the rate of β-oxidation of fatty acids. In the liver of rats, ACC protein was diminished by fasting and increased following fasting and refeeding with a high carbohydrate diet (136, 137). In chickens, ACC mRNA and protein in the liver increased markedly after hatch in growing chicks (138). Many studies indicate that this developmental regulation in growing chicks is associated with changes in nutrition, from relatively high-fat, low-carbohydrate nutrition in chick embryos to relatively low-fat, high-carbohydrate nourishment in growing chicks (139).

A high-carbohydrate, low-fat diet also affects the gene expression of other lipid metabolism-associated factors in the liver. For example, feeding previously 24-hour fasted mice a high-carbohydrate, low-fat diet led to increased hepatic mature SREBP-1c expression 4- to 5-fold above non-fasted levels (140). Collectively, these results suggest that factors associated with fatty acid and lipid synthesis are up-regulated in the liver and adipose tissue of animals that consume a relatively high-carbohydrate, low-fat diet, reflecting the partitioning of energy from glucose to other physiological pathways besides direct oxidation.

Dietary protein. Dietary protein quantity profoundly affects animal growth and body composition. An increase in dietary protein from 20% to 33% was associated with decreased abdominal fat, and enhanced body weight gain and breast muscle yield in 28-day-old commercial broilers (fed after day of hatch) (141). In addition, a high-protein diet (23% vs 17% or 17% vs 13%) also decreased abdominal fat and increased body weight and breast meat yield in chicks from lines selected for high and low abdominal fatness, compared with birds that were fed a low-protein diet for 9 or 7 weeks post-hatch (142, 143). However, feeding diets with varying protein levels (from 18 to 28%) for the first week post-hatch did not alter carcass fat deposition in 7 day-old broiler chick (100). Thus, similar to effects of dietary fat, dietary protein probably affects carcass quality of chickens more dramatically at a later age.

Mohiti-Asli et al. (2012) reported that feeding increased dietary protein (17.4 vs. 14.5 %) for 12 weeks was associated with reduced hepatic malic enzyme activity and decreased abdominal fat weight and hepatic and plasma TAG concentrations in laying hens, suggesting that malic enzyme is important in regulating the rates of de novo fatty acid synthesis in the avian liver (144). In addition, 6 and 24 h of feeding a high protein diet (40 vs 22 %) decreased malic enzyme activity and total liver lipid content in 6 and 7 day-old chicks (145). The activity of hepatic malic enzyme may be correlated with rates of lipid synthesis, since it has been suggested that malic enzyme in addition to the dehydrogenases of the glucose monophosphate shunt may provide NADPH for lipogenesis in the liver (146, 147).

Amino acid supplementation. Depending on the genetic background and age, supplemental essential amino acids are provided in the poultry diet at varying concentrations. Amino acids exert various biological functions in addition to being an energy source or constituent of proteins. Several studies have evaluated effects of dietary amino acid supplementation on adipose tissue

physiology of birds and mammals. Arginine is a protein constituent that acts as an insulin and growth hormone secretagogue, as well as a precursor for creatine, nitric oxide and polyamine synthesis (148, 149). When arginine was supplemented in the drinking water at 1.51% for 12 weeks, relative weights of specific muscles and percentage of brown fat were increased while white adipose tissue pad weights were reduced by 30%, compared with control rats that were provided 2.55% L-alanine (isonitrogenous control) (150). One of the explanations was that arginine-associated increases in nitric oxide production led to increased mitochondrial biogenesis, which in turn generated more oxidative activity and heat production. Consistent with this hypothesis, Tan et al. (2011) showed that in pigs that consumed diets supplemented with 1.0% arginine (in a diet that met all nutrient requirements) there was increased skeletal muscle mass, decreased adipose tissue, and increased lipid content in the longissimus dorsi. The expression of FAS mRNA in muscle and HSL mRNA in adipose tissue was increased, whereas LPL, glucose transporter-4 and ACC-α mRNAs were decreased in adipose tissue, as compared with control pigs (2.05 % L-alanine) (151). These results from rat and pig studies indicate that dietary arginine has the capacity to shift nutrient partitioning whereby lipogenesis is favored in muscle while lipolysis is enhanced in adipose tissue.

Birds are unable to synthesize arginine because the urea cycle is not functional, with uric acid being the primary form of nitrogen excretion in avians (152). At an early age, chicks have a markedly acute need for dietary arginine as it is also possibly involved in immune system development and defending against early microbial challenges (153). The effect of arginine supplementation on fat deposition has been inconsistent in chickens. Mendes et al. (1997) reported that from 21 to 42 days of age increasing dietary arginine:lysine ratios (from 1.1:1 to 1.4:1) reduced abdominal fat content (154). In 6-week-old ducks that were fed 1% arginine for

the first 42 days post-hatch, there was reduced carcass fat deposition and smaller abdominal adipocytes, which authors attributed to a decrease in hepatic lipogenic enzyme activity, suggesting that there were fewer circulating fatty acids available for lipid deposition in adipose tissue (155). However, 0.4% arginine supplementation (in a diet that met all nutrient requirements) only during the starting phase (1 to 21 d) did not affect abdominal fat deposition in 6-week-old chickens (152). The lack of effect of dietary supplemental arginine could be related to the duration of feeding or inclusion rate of arginine in the diets.

Lysine is one of the key amino acids for protein synthesis and muscle deposition. It is also important for immune system function in response to infection, a lysine deficiency hindering antibody response and cell-mediated immunity in chickens (156). Dietary lysine levels also affect carcass composition. In 6-week-old chickens that were fed diets with lysine levels 10% above or below the requirement levels, abdominal fat deposition was significantly increased in chickens fed both diets, which could be associated with a dietary amino acid imbalance, resulting in excess amino acids being catabolized, carbons then used as gluconeogenic substrate and resulting glucose catabolized and converted into fatty acids, and deposited into adipose tissue (157).

In poultry diets, methionine is usually the first limiting amino acid. Five-week-old chickens fed 0.60% methionine (total methionine level in the diet) for 4 weeks had reduced abdominal fat compared with chickens fed 0.40% methionine (both met methionine requirements) (158). However, when a dietary methionine deficiency was imposed, body fat deposition was increased at 6- and 8-weeks of age in broilers (159).

Other dietary factors. Soy isoflavones are reported to be involved in the regulation of physiological activities in a variety of tissues or organs, including adipose tissue, liver and

skeletal muscle (160, 161). Takahashi and Ide (2008) reported that dietary supplementation of 0.5 or 4 g/kg diet soy isoflavones increase fatty acid oxidation in rat liver by up-regulating PPARγ and uncoupling protein expression (162). In chickens, dietary soy isoflavone supplementation (0.70 and 1.73 g/kg diet vs 0.35 g/kg diet in the starter diet) was associated with increased breast muscle yield but did not affect abdominal fat percentage of body weight in 52-day-old broiler chickens (163).

As a trimethyl derivative of glycine, betaine may decrease the dietary requirement for other methyl donors including choline and methionine. Dietary supplementation of betaine reduced abdominal fat deposition in broiler chickens (164, 165). Xing et al. (2011) hypothesized that dietary supplementation of 0.1% betaine decreases abdominal fat through a down-regulation in the expression of LPL and FAS mRNA in abdominal fat compared with basal diet feeding, as observed in 66-day-old broilers (166).

Probiotics have been used to prevent overgrowth of undesirable microbes in the gastrointestinal tract and favor the establishment of benign bacterial species. Dietary supplementation of some strains of probiotics (*Bacillus subtilis* or *Bacillus cereus toyoi*) decrease abdominal fat accumulation in chickens (167) and Japanese quail (168). Reduced abdominal fat in chickens after dietary probiotic supplementation may be partially explained by decreased ACC activity in the liver (168).

Conclusions and implications

Adipose tissue development is mediated by a variety of factors, including nutritional, hormonal and transcription factors. There are a variety of transcription factors that regulate adipogenesis and among them PPARγ is considered to be the "master" regulator. There have been

advancements in understanding adipose tissue development in chickens, although the molecular and cellular mechanisms are not fully elucidated. Feed constitutes a large proportion of total broiler production costs, thus, maximizing the efficiency of feed utilization is important from an economical perspective. Understanding the cellular pathways governing adipose tissue development and how these pathways are influenced by dietary factors may contribute to practical strategies to improve poultry production efficiency and provide novel insights for biomedical research. We reviewed papers describing how altering the amount and composition of fat and protein in the diet affects abdominal fat accumulation in broilers. There is evidence to suggest that replacing SFAs with PUFA, or supplementing additives in broiler breeder diets may reduce abdominal fat deposition thus improving reproductive performance. Broiler chickens have been selected for rapid growth, and for broiler breeders egg production is inversely proportional to body weight. Thus, nutritional manipulations may improve the fitness and welfare of broiler chickens by increasing reproductive effectiveness and reducing the incidence of musculoskeletal diseases, metabolic disorders and mortality. Such practices will also improve the welfare of broiler breeders as dedicated programs of feed restriction are applied to maintain proper body weight. In conclusion, understanding how diet composition affects adipose tissue development has many economical and health and welfare implications for poultry production.

Table 2-1. Some factors affecting adipose development in chickens.

Factors	Mechanisms[1]	Citations
Fat		
Sunflower oil	Hepatic FAS -	Sanz et al., 1999
	CPT I and L3HOAD in the heart +	Sanz et al., 2000
Linseed oil	Fatty acid β-oxidation -	Sanz et al., 2000
Fatty acids		
Oleic acid	FABP4 and C/EBP$_\beta$ -	Regassa et al., 2013
Conjugated linoleic acid	PPARγ mRNA expression -	Ramiah et al., 2014
Polyunsaturated fatty acid	Fat synthesis -	Sanz et al., 2000
	Fatty acid oxidation +	
Medium-chain fatty acid	Not fully understood	Wang et al., 2015
Carbohydrates	FAS synthesis +	Back et al., 1986
Protein	Hepatic malic enzyme -	Mohiti-Asli et al., 2012
Arginine	Hepatic lipogenic enzyme -	Wu et al., 2011
Lysine	Dietary amino acid imbalance	Nasr et al., 2012
Methionine	Not fully understood	Takahashi et al., 1995
Other factors		
Soy isoflavones	Unknown	
Betaine	LPL and FAS mRNA expression -	Xing et al., 2011
Probiotics	Lipid biosynthesis -	Homma and Shinohara, 2004
	Fatty acid catabolism +	

[1] +, increased; -, decreased. Abbreviations: FAS, fatty acid synthase; CPT I, carnitine palmitoyltransferase I; L3HOAD, L-3-hydroxyacyl-CoA dehydrogenase; FABP4, fatty acid binding protein 4; C/EBP$_\beta$, CCAAT/enhancer binding protein-β; PPARγ, peroxisome proliferator-activated receptor γ; LPL, lipoprotein lipase; ACC, acetyl-CoA carboxylase.

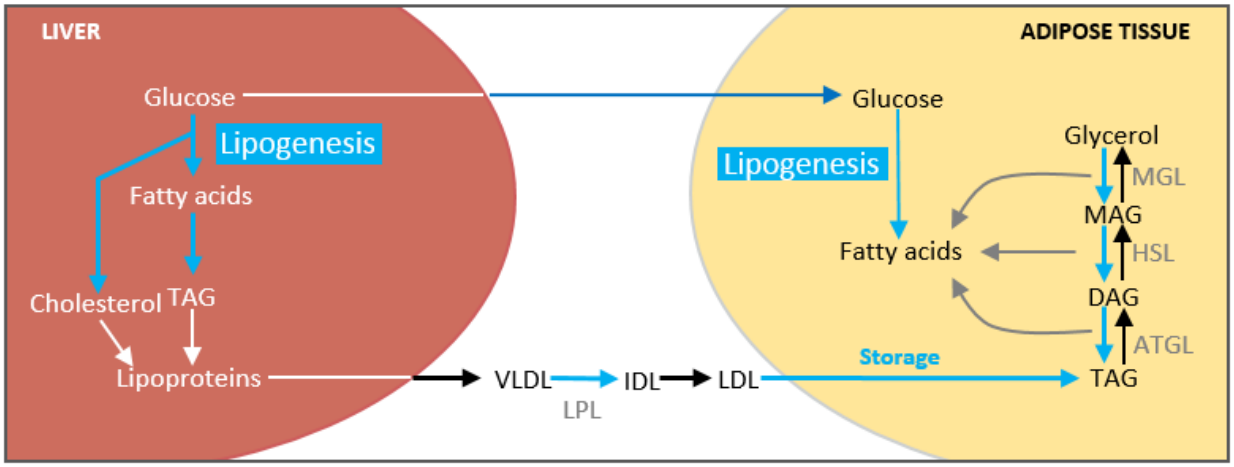

Figure 2-1. The liver and adipose tissue are the two main sites of de novo fatty acid synthesis in higher vertebrates. In avian species, the liver is the main site of de novo lipogenesis, where glucose is catabolized to acetyl coA, which is then converted into fatty acids and cholesterol. In the liver, cholesterol and triacylglycerols (TAGs) are incorporated into very low density lipoprotein (VLDLs) to be transported to other tissues via the circulation. Lipoprotein lipase (LPL), anchored to endothelial cells lining tissues throughout the body, facilitates fatty acid delivery to peripheral tissues by hydrolyzing ester bonds of TAG. As more TAGs are removed, VLDL change in composition and become intermediate density lipoproteins (IDLs), which are then converted to low density lipoprotein (LDLs) as more fatty acids are sequestered. In the lipid droplet of the adipocyte, lipolysis of stored TAG is catalyzed sequentially by adipose triglyceride lipase (ATGL), hormone sensitive lipase (HSL) and monoglyceride lipase (MGL). ATGL breaks down TAG to diacyglycerol (DAG) and one fatty acid. HSL and MGL catalyze fatty acid removal from DAG and monoacyglycerol, respectively. Parts of the figure are adapted (169).

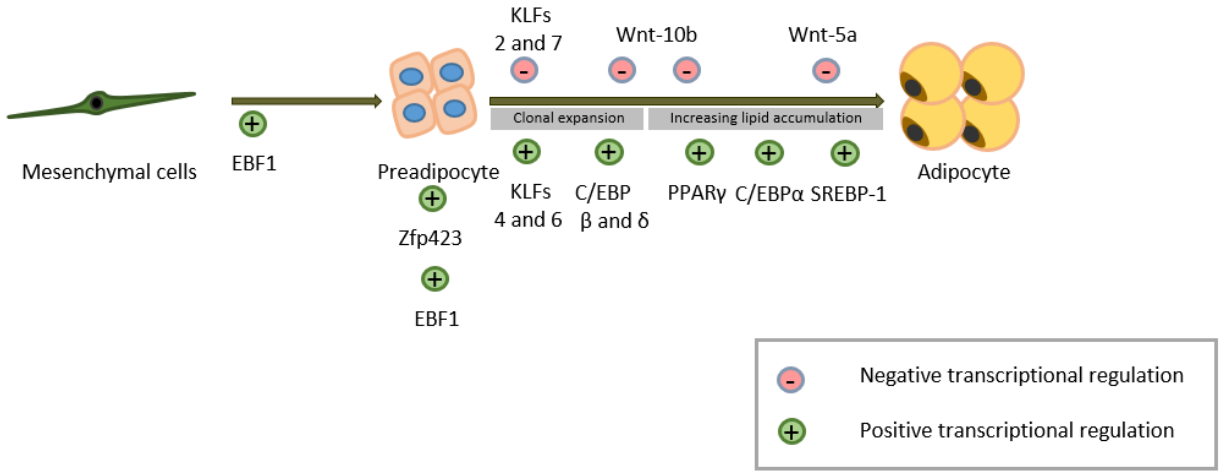

Figure 2-2. Transcription factors that orchestrate adipocyte development. Parts of the figure are adapted (170, 171). Abbreviations: EBF1, early B cell factor 1; Zfp423, zinc finger protein 423; KLFs, Krüppel-like transcription factors; C/EBPs, CCAAT/enhancer binding proteins; PPARγ, peroxisome proliferator-activated receptor γ; SREBP-1, sterol regulatory element binding protein 1.

Chapter 3: The effects of dietary macronutrient composition on lipid metabolism-associated factor gene expression in the adipose tissue of chickens are influenced by fasting and refeeding

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Abstract

Broiler chickens are compulsive feeders that become obese as juveniles and are thus a unique model for metabolic disorders in humans. However, little is known about the relationship between dietary composition, fasting and refeeding and adipose tissue physiology in chicks.

Our objective was to determine how dietary macronutrient composition and fasting and refeeding affect chick adipose physiology during the early post-hatch period. Chicks were fed one of three isocaloric diets after hatch: high-carbohydrate (HC; control), high-fat (HF; 30% of ME from soybean oil) or high-protein (HP; 25% vs. 22% crude protein). At 4 days post-hatch, chicks were fed (continuous ad libitum access to food), fasted (3 h food withdrawal), or refed (fasted for 3 h and refed for 1 h). Subcutaneous, clavicular, and abdominal adipose tissue was collected for histological analysis and to measure gene expression, and plasma to measure non-esterified fatty acid (NEFA) concentrations (n = 6-10 per group). Adipose tissue weights were reduced in chicks that were fed the HP diet and adipocyte diameter was greater in the adipose tissue of chicks that ate the HF diet. Consumption of diets differing in protein and fat content also affected gene expression; mRNAs encoding fatty acid binding protein 4 and a lipolytic enzyme, monoglyceride lipase, were greater in chicks fed the HC and HF than HP diet in all three adipose tissue depots. Fasting influenced gene expression in a depot-dependent manner, where most fasting and

refeeding-induced changes were observed in the clavicular fat of chicks that consumed the HC diet. Fasting increased plasma NEFA concentrations in chicks fed the HC and HP diets.

The decreased adipose tissue deposition in chicks fed the HP diet is likely explained by decreased rates of adipogenesis. Consumption of the HF diet was associated with greater adipose tissue deposition and larger adipocytes, likely as a result of greater rates of adipocyte hypertrophy. The depot-dependent effects of diet and fasting on gene expression may help explain mechanisms underlying metabolic distinctions among subcutaneous and visceral fat depots in humans.

Key words: Dietary macronutrients, fasting, adipose tissue, mRNA abundance, chicks

Introduction

Intensive selection for growth rate and meat yield in broiler chickens has led to correlated increases in voluntary food consumption, fat deposition and incidence of metabolic disorders in breeders (54). Thus, chickens may serve as a model to better understand the genetic and molecular basis for metabolic disorders in humans. In mammals, it is well-known that subcutaneous fat can prevent other tissues from accumulating excessive lipids that can cause lipotoxicity, therefore acting as a buffer for the daily incorporation of dietary fat (50). It is also considered to be metabolically benign as compared to other anatomical depots. However, visceral fat (body fat stored within the abdominal cavity) is associated with metabolic disorders (51), yet little is known about whether such differences exist in avian species.

Dietary macronutrient composition not only affects appetite in birds and mammals but also regulates adipose tissue physiology. When mice are fed a high-fat (HF) diet, hypertrophy dominates in visceral fat whereas hyperplasia dominates in subcutaneous fat as both depots

expand in response to excess caloric intake (172). However, little is known about the effects of dietary macronutrient composition on adipose tissue development in avian species. We recently reported that a HF diet enhanced the sensitivity to the effects of exogenous neuropeptide Y (NPY) on food intake in chicks and that NPY in turn led to increased food intake in chicks that consumed a high-protein (HP) and high-carbohydrate (HC), but not HF diet (173).

In response to food deprivation and refeeding, a complex array of adaptive metabolic changes are triggered that mediate dynamic alterations in appetite regulation, energy storage and expenditure in peripheral tissues in response to the changes in energy availability. The effects of fasting and refeeding on the gene expression of metabolism-associated genes in different tissues has been extensively studied in both birds and mammals, however, there is little known about the effects of diet on molecular changes in the adipose tissue of chicks. Therefore, the purpose of this study was to evaluate effects of dietary macronutrient composition and fasting and refeeding on adipose tissue physiology in broilers chicks during the first 4 days post-hatch.

Materials and methods

Animals

All animal protocols were approved by the Institutional Animal Care and Use Committee at Virginia Tech and animals were cared for in accordance with the Guide for the Care and Use of Laboratory Animals. Day of hatch Cobb-500 broiler chicks were obtained from a local hatchery and housed in electrically heated and thermostatically controlled cages with ad libitum access to feed and water. The temperature was 30 ± 2 ℃ with 50 ± 5% relative humidity and 24 h of light. Diets were formulated to be isocaloric (3,000 kcal/kg) as shown in Table 3-1 and mixed at Augusta Cooperative Feed Mill (Staunton, Virginia, USA). The HP diet was formulated to

contain 25% crude protein and the HF diet to have 30% of the ME derived from calories in soybean oil. Crude protein and fat values were experimentally verified for all diets (Table 3-1).

Chicks were randomly assigned to one of the three diets at day of hatch, with ad libitum access to food and water. At 4 days post-hatch, chicks from each diet were randomly assigned to one of three treatments: fasting (3 h), refeeding (1 hour of ad libitum access to food after 3 h of fasting) and feeding (continuous ad libitum access to food), with n = 10 chicks per group. Chicks were euthanized, sexed by visual inspection of gonads, and tissues collected as described below.

Adipose tissue depot weights and histology.

Subcutaneous, clavicular, and abdominal adipose tissue samples were collected from n = 10 chicks as described (174), from each of the three dietary groups (HC, HF, and HP) at 4 days post-hatch (all continuously fed). Adipose tissue depots were weighed and weights were converted into a percentage of the chick's body weight. Samples were prepared for histology as described (174). Samples were rinsed in phosphate-buffered saline, submerged in neutral-buffered formalin and incubated overnight on a rocking platform at 4℃. Samples were then dehydrated in a graded ethanol series, paraffin embedded, sectioned at 5 μm and mounted. One section was mounted per slide with two slides (at least 200 μm apart) per sample. Only samples from chicks assigned to the "fed" treatment within each dietary group were used for histology. Slides were stained with hematoxylin and eosin and images were captured with a Nikon Eclipse 80i microscope and DS-Ri1 color camera and analyzed using NIS-Elements Advanced Research Software (Nikon). The density, diameter, and area of all adipocytes within the field of an image were measured under 20x magnification. The threshold method was used to count adipocytes. Adipocytes were treated as binary objects with the restriction that measurements must exceed 100 μm^2. Size distributions in each image were also analyzed.

Plasma NEFA concentrations.

Approximately 200 μL of blood was collected from the trunk (n = 10 chicks) via capillary blood collection tubes (Microvette®) immediately following euthanasia and decapitation. After collection, samples were centrifuged at 2,000 x g at room temperature and plasma isolated. Plasma NEFA concentrations were measured using the NEFA-HR2 kit (Wako Diagnostics) according to the manufacturer's instructions. Absorbance was measured at 550 nm using an Infinite M200 Pro multi-mode plate reader (Tecan). Sample concentration was calculated using the following formula: Sample Concentration = Standard Concentration × (Sample Absorbance) / (Standard Absorbance). Units for the concentrations are reported as mEq/L.

Total RNA isolation and real-time PCR

Adipose tissue samples were collected (n=10 per group) and submerged in RNAlater (Qiagen). Tissues were homogenized in 1 mL Isol RNA Lysis reagent (5-Prime, Gaithersburg, MD, USA) using 5 mm stainless steel beads (Qiagen, Valencia, CA, USA) and a Tissue Lyser II (Qiagen) for 2 × 2 min at 25 Hz. Total RNA was separated following the manufacturer's instructions (5-Prime) and following the step of addition to 100% ethanol, samples were transferred to spin columns and further purified using Direct-zol RNA Kits (Zymo Research) with the optional RNase-free DNase I (Zymo Research) treatment. Total RNA integrity was verified by agarose-formaldehyde gel electrophoresis and concentration quantified and purity assessed by spectrophotometry at 260/280/230 nm with a Nanophotometer Pearl (IMPLEN, Westlake Village, CA, USA). Single-strand cDNA was synthesized with a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) and 200 ng of total RNA, following the manufacturer's instructions. Reactions were performed under the following conditions: 25 ℃ for 10 min, 37 ℃ for 120 min and 85 ℃ for 5 min. Primers for real time PCR were designed with Primer Express

3.0 software (Applied Biosystems), and validated for amplification efficiency (within 5% of reference gene) before use (Table 3-2). Real-time PCR was performed in duplicate in 10 µl volume reactions that contained 5 µl Fast SYBR Green Master Mix (Applied Biosystems), 0.25 µL each of 5 µM forward and reverse primers, and 3 µl of 10-fold diluted cDNA using a 7500 Fast Real-Time PCR System (Applied Biosystems). PCR was performed under the following conditions: 95 ℃ for 20 s and 40 cycles of 90 ℃ for 3 s plus 60 ℃ for 30 s. A dissociation step consisting of 95 ℃ for 15 s, 60 ℃ for 1 min, 95 ℃ for 15 s and 60 ℃ for 15 s was performed at the end of each PCR reaction to ensure amplicon specificity.

Statistical analysis

The real time PCR data were analyzed using the ΔΔCT method, where ΔCT = CT target gene − CT actin, and ΔΔCT = ΔCT target sample − ΔCT calibrator (175). The average of clavicular fat from chicks fed the HC diet that received the "fed" treatment was used as the calibrator sample. The fold difference (relative quantity; RQ) was calculated as $2^{-\Delta\Delta CT}$. Analysis of variance (ANOVA) was performed for adipose depot weight, percent weights, adipocyte area and diameter, NEFA concentrations, and RQ values using the Fit Model platform of JMP Pro11 (SAS Institute, Cary, NC). Because sex and interactions between sex and diet did not significantly influence any of the traits measured in this study, sex was excluded from the statistical models. For weights and morphometric measurements, the statistical model included the main effects of diet, adipose tissue depot, and their interaction. Post hoc pairwise comparisons were carried out with Tukey's test. For RQ data (analyzed within depot) and NEFAs, the model included the main effects of diet and fasting treatment and the interaction between them. The interaction on NEFAs was separated with Tukey's test. Significant interactions from real time PCR data were separated with the Slice function of JMP and effects sliced within

dietary group for each gene. Significant dietary effects were further analyzed by second ANOVAs with Tukey's test to separate the means. All data are presented as means ± SEM. Differences were considered significant at $P < 0.05$.

Results

Adipose tissue depot weights and histology

At day 4 post-hatch, body weights were not different among groups and chicks fed HC and HF diets had greater fat depot weights ($P = 0.0001$) than chicks fed the HP diet (Table 3-3), even when expressed as a percentage of body weight ($P = 0.0002$). The subcutaneous fat was heaviest, clavicular fat intermediate, and abdominal fat the lightest ($P = 0.0001$). This was also observed when depots were expressed as a percentage of body weight ($P = 0.0001$). Adipocyte diameter was greater ($P < 0.05$) in chicks fed the HF diet than in chicks fed the HC or HP diet (Table 3-4). Adipocyte area and diameter were greatest in subcutaneous fat, intermediate in clavicular and smallest in abdominal fat ($P < 0.0001$).

More than 64% and 59% of adipocytes were 10 to 25 μm in mean diameter in the subcutaneous fat of chicks fed the HC and HP diets, while only 49% of the adipocytes were 10 to 25 μm in the subcutaneous fat of chicks fed the HF diet (Figure 3-1A). Most of the adipocytes (> 75%) were 10 to 25 μm in mean diameter in the clavicular fat of chicks fed the HC and HP diets (Figure 3-1B). However, only 55% of the adipocytes were smaller than 25 μm in mean diameter in the clavicular fat of chicks fed the HF diet. In the abdominal fat, most of the adipocytes (> 91%) were 10 to 25 μm in mean diameter at day 4, irrespective of the diet (Figure 3-1C). The gross histology (Figure 3-2) also suggests that there were many highly-vascularized clusters of what

might be preadipocytes in the abdominal fat (Figure 3-2C), but not in the other two adipose depots of all chicks, independent of diet.

Plasma NEFA concentrations

There was an interaction of feeding treatment and diet on plasma NEFA concentrations (P = 0.02). Plasma NEFAs were greater in fasted than fed or refed chicks that consumed the HC and HP diets (P = 0.02; Figure 3-3). Plasma NEFAs were not affected by fasting or refeeding in chicks that consumed the HF diet. Plasma NEFAs were not different among dietary groups.

mRNA abundance in subcutaneous adipose tissue

The mRNA abundance results for subcutaneous, clavicular, and abdominal adipose tissue are summarized in Tables 2-5, -6, and -7, respectively. Significant two-way interactions are displayed graphically. There were interactions of diet and feeding treatment on CCAAT/enhancer-binding protein alpha (*C/EBP*α) and NPY receptor 2 (*NPYR2*) in subcutaneous adipose tissue (Figure 3-4). *C/EBP*α expression was greater in fed than fasted or refed chicks that consumed the HC diet, and greater in fed and refed than fasted chicks that consumed the HP diet, whereas in chicks that consumed the HF diet, expression was greatest in fed, intermediate in refed, and lowest in fasted chicks ($P < 0.05$; Figure 3-4A). Expression of NPYR2 was greater in fed than fasted or refed chicks that consumed the HC diet, greater in fed than refed chicks that consumed the HF diet, and was similar across treatments in chicks that consumed the HP diet ($P < 0.05$; Figure 3-4B).

There were also main effects of diet and feeding condition. Expression of fatty acid binding protein 4 (*FABP4*) was greater in chicks that consumed the HC than the HP diet (P = 0.02; Table 3-5). Expression of 1-acylgylcerol-3-phosphate O-acetyltransferase 9 (*AGPAT9*), peroxisome

proliferator-activated receptor gamma (*PPARγ*), and sterol regulatory element-binding transcription factor 1 (*SREBP1*) was greater in the subcutaneous adipose tissue of fed than fasted or refed chicks ($P < 0.05$). *C/EBPβ* mRNA was greater in fasted than fed or refed chicks ($P < 0.05$), while Krüppel-like factor 7 (*KLF7*) was greatest in fasted, intermediate in fed, and lowest in the subcutaneous fat of refed chicks ($P < 0.05$).

mRNA abundance in clavicular adipose tissue

In clavicular fat, *AGPAT9*, *PPARγ*, *SREBP1*, adipose triglyceride lipase (*ATGL*), and *NPYR2* mRNA quantities were affected by the interaction of feeding treatment and diet (Figure 3-5). Expression of *AGPAT9* (Figure 3-5A), *SREBP1* (Figure 3-5C), and *ATGL* (Figure 3-5D) showed similar expression patterns, where in chicks that consumed HC but not the other two diets, mRNA was greater in fed than fasted or refed chicks ($P < 0.05$). The quantities of *PPARγ* (Figure 3-5B) and *NPYR2* (Figure 3-5E) mRNA showed similar responses, where in all three dietary groups there was greater expression in fed vs. fasted or refed chicks with a greater difference in chicks that consumed the HC diet ($P < 0.05$).

There were multiple main effects of diet and feeding state in clavicular fat. Perilipin1 (*PLIN1*), *FABP4*, and monoglyceride lipase (*MGLL*) mRNAs were greater in the clavicular fat of chicks that consumed the HC and HF than HP diet ($P < 0.05$; Table 3-6). Expression of *FABP4* and *PLIN1* mRNA was also greater in fed than refed chicks ($P < 0.05$), while abundance of *C/EBPβ* and acyl-CoA dehydrogenase long chain (*ACADL*) mRNA was greater in fasted than fed chicks ($P < 0.05$). There was greater expression of comparative gene identification-58 (*CGI-58*) in fed chicks than fasted or refed chicks ($P < 0.05$). The expression of Krüppel-like factor 7 (*KLF7*) and *NPY* mRNA was greater in fasted than fed or refed chicks ($P < 0.05$).

mRNA abundance in abdominal adipose tissue

The *CGI-58* and *NPYR2* mRNA quantities were affected by the interaction of diet and feeding treatment in abdominal fat (Figure 3-6). In chicks that consumed the HC diet, expression of CGI-58 was greater in refed than fasted or fed chicks, while fasting and refeeding had no effects on expression in chicks that consumed the HP or HF diet ($P < 0.05$; Figure 3-6A). In chicks that ate the HC diet, *NPYR2* mRNA was greater in fasted than fed chicks, and for chicks that ate the HP diet, expression was greater in the abdominal fat of fed than refed chicks ($P < 0.05$; Figure 3-6B).

Similar to the other depots, there were multiple main effects in abdominal fat. The abundance of *C/EBPβ* and *MGLL* mRNA was greater in the HC than HP-fed chicks ($P < 0.05$; Table 3-7). Expression of *KLF7* mRNA was greater in HF than HP-fed chicks, whereas *ACADL* was greater in HP than HF-fed chicks ($P < 0.05$). There were also many main effects of feeding treatment on mRNA abundance in abdominal fat. Expression of *AGPAT9* was greater in fed than refed chicks ($P < 0.05$). Expression of *C/EBPα* was greatest in fed, lowest in fasted, and intermediate in refed chicks, while *C/EBPβ* was only greater in fasted than fed chicks ($P < 0.05$). The abundance of *KLF7* mRNA was greater in fasted than fed or refed chicks ($P < 0.05$). Expression of *PPARγ* was greatest in fed, intermediate in refed, and lowest in the abdominal fat of fasted chicks ($P < 0.05$). Expression of *MGLL* was greater in fed and fasted than refed chicks ($P < 0.05$). The abundance of *SREBP1* mRNA was greatest in fed, intermediate in fasted, and lowest in refed chicks and *NPY* was greater in fed than fasted or refed chicks ($P < 0.05$).

Discussion

The current obesity epidemic and projected increases in the prevalence of obesity worldwide necessitate the development of novel research models that provide insights on the pathogenesis

of obesity at the cellular and molecular level. This research revealed that not only do different anatomical regions of fat develop differently in chicks, but that dietary fat and protein quantity dramatically influence adipocyte development and lipid remodeling. Three hours of fasting was sufficient to induce a rise in plasma non-esterified fatty acids (NEFAs), however the effect was diet-dependent, and gene expression changes were detected in a diet and adipose tissue depot-dependent manner, further illustrating the complex relationship between diet composition, nutritional state, and anatomical depot-specific physiology. These findings highlight the importance of understanding metabolic differences among subcutaneous and visceral adipose tissue depots and the utility of chick as a model to explore the molecular basis for such differences in humans.

At 4 days post-hatch, there was less adipose tissue deposition in chicks that consumed the HP diet. Feeding more dietary protein (from 18 to 28%) for the first week post-hatch did not affect body fat content in 7 day-old broiler chicks (100), although abdominal fat content was decreased when dietary protein was increased at a later age (142, 143). In rats, both long term (6 months) or short term (3 weeks) feeding of a HP diet reduced total white adipose tissue (WAT) weights (176, 177). This may be partially explained by the reduced energy intake from consuming the HP diet, as protein induces greater sensory-specific satiety compared with carbohydrates (178, 179). However, based on our previous publications, the HP diet does not seem to have a satiating effect on food intake in chicks (173, 180), although the protein content of the HP diet used in the present study was lower as compared to our previous studies (25 vs. 30 % CP), and there was no difference in food intake between the HP and other diets during the first 4 days post-hatch (181).

Differences in adipose tissue weight can be partly explained by the gene expression results. In the adipose tissue of chicks consuming the HP diet, there was reduced expression of *FABP4*

(subcutaneous and clavicular), *SREBP1* (clavicular) and *C/EBPβ* (abdominal) in comparison with the HC diet, and *FABP4* (clavicular) and *KLF7* (abdominal) compared with the HF diet. The SREBP1 and C/EBPβ are key transcription factors during the early stages of adipogenesis that coordinate the transcriptional regulation of a variety of adipocyte metabolism-associated genes (58, 182, 183). The biological function of FABP4 involves the binding and transport of fatty acids from cell membranes into adipocytes (184). The KLF7 is a member of the Krüppel-like transcription factor family that promotes chicken preadipocyte proliferation but inhibits its differentiation (71). Collectively, these results indicate that there might be reduced rates of preadipocyte proliferation and differentiation and associated fatty acid incorporation into triacylglycerols in adipose tissue from chicks fed the HP diet, resulting in less adipose tissue deposition in a depot-specific manner. That adipocyte size and size distribution were similar between HP and HC diet-fed chicks also supports that in the HP diet-fed chicks the reduced weight of the adipose tissue was due to reduced numbers of adipocytes, possibly from less adipogenesis, compared with HC diet-fed chicks.

In mice more than 6 weeks old, 60 days of feeding a HF diet revealed that visceral fat expanded predominantly by adipocyte hypertrophy, whereas subcutaneous fat expanded by adipocyte hyperplasia (172). Increased intra-abdominal/visceral fat is associated with a greater risk of developing metabolic diseases, whereas increased subcutaneous fat in the thighs and hips represents little or no risk (185). A relative lack of progenitor cell activity may be the reason why adipose depots such as visceral accumulate hypertrophic, dysfunctional adipocytes and are consequently associated with a higher risk of metabolic diseases (172). Our results revealed that consumption of the HF diet was associated with larger adipocytes. Consistent with our previous research (174), fat depot weight and adipocyte area and diameter were greater in subcutaneous

and clavicular than abdominal fat at day 4 post-hatch. The high percentage of small adipocytes (> 91% were 10 to 25 μm) in the abdominal fat along with more of what appear to be preadipocyte clusters may suggest that a combination of hyperplasia and hypertrophy contribute to the adipose tissue development of abdominal fat, the depot that develops at a more rapid rate post-hatch than other depots in broiler chickens (174).

Expression of genes involved in the early stage of adipogenesis, the commitment of mesenchymal stem cells to form new adipocytes, was downregulated in the abdominal fat of broiler chickens after 5 h of fasting (186). In the present study, fasting downregulated the mRNA abundance of *PPARγ*, *SREBP1*, and *C/EBPα* (except in the clavicular fat) and upregulated *C/EBPβ* and *KLF7* mRNA in all three adipose tissue depots. Similarly, 4 h of fasting decreased adipose tissue *PPARγ* and *SREBP1* mRNA in 13 day-old chicks (187). These effects are also consistent with effects of 4 and 8 h of fasting on *PPARγ* and *SREBP1* mRNA in the adipose tissue of rats (188, 189). In rodents, PPARγ and SREBP1 are considered to be lipogenic transcription factors in the adipose tissue, as de novo lipogenesis occurs at a greater rate in the adipose tissue of rodents compared to humans or birds. The PPARγ is able to activate glucose transporter 4 expression, indicative of increased fatty acid synthesis from glucose (190). SREBP1 regulates the expression of key genes involved in lipid and glucose metabolism, such as *FAS* and acetyl-coenzyme A carboxylase (191, 192). However, unlike many mammals in which adipocytes are the main site of *de novo* lipogenesis, *de novo* lipogenesis is considered to take place almost exclusively in the liver of avian species, similar to humans (19). Therefore, decreased *PPARγ* and *SREBP1* mRNA expression in chicken adipose tissue after fasting does not necessarily suggest reduced lipogenesis in response to fasting, but may reflect a downregulation of triacylglycerol synthesis in the absence of available substrate.

Except for its roles in lipogenesis, the activation of PPARγ also promotes terminal differentiation, which is achieved through the induction of a variety of differentiation-dependent genes that are crucial for fatty acid uptake and storage, such as *FABP4*, *LPL* and others (58). In addition to promoting adipocyte differentiation, PPARγ also plays an important role in adipocyte hypertrophy in the adipose tissue, upregulating expression of enzymes such as LPL in the adipocyte (193). As a critical adipogenic regulator, C/EBPα also induces the expression of differentiation-associated factors (58).

Although the expression of genes such as *PPARγ* and *SREBP1* had similar expression patterns in each adipose depot in response to fasting, there were some depot-dependent changes. Fasting decreased *AGPAT9* mRNA in subcutaneous fat, decreased *NPY* in abdominal fat, while it increased *NPY* expression in clavicular fat, and decreased *NPYR2* in clavicular and subcutaneous fat. The *AGPAT9* encodes an enzyme that catalyzes the initial step of *de novo* triacylglycerol synthesis (194). Sympathetic neuron-derived NPY and activation of its receptor NPYR2 promote adipocyte proliferation and differentiation (91), and knockout of *NPYR2* resulted in reduced body weight gain and adiposity (92). Increased lipolysis during fasting is expected to be accompanied by a corresponding reduction in triacylglycerol synthesis/lipogenesis. Collectively, these changes indicate that transcriptional regulation of adipogenesis-associated factors is altered in response to fasting in order to down-regulate preadipocyte differentiation and decrease adipocyte hypertrophy in a depot-dependent manner.

In rats, circulating NEFAs increased after 4 h of fasting and peaked at 8 h, however, *ATGL* mRNA was increased in retroperitoneal WAT after 24 but not 8 h of fasting (189). Thus, the rapid increase in circulating NEFAs under fasting conditions is most likely explained by an upregulation of enzyme activity, occurring prior to changes induced at the transcriptional or

translational level (189). Adipose triglyceride lipase and hormone sensitive lipase (HSL) are the major enzymes involved in triacylglycerol catabolism, being responsible for more than 95% of the hydrolase activity in murine WAT (195). However, no HSL orthologue has been identified in the chicken genome (45), suggesting an even more important role of ATGL in lipolysis in chickens. In the present study, *ATGL* mRNA was not upregulated after 3 h fasting in any of the three adipose depots, indicating that lipolysis might be promoted by an upregulation of the enzyme at the protein level, similar to mammalian studies, although it should be noted that 3 h of fasting increased plasma NEFA concentrations only in chicks that consumed the HC and HP diets.

Under basal conditions, PLN1 may inhibit lipolysis by blocking the binding of lipase to triacylglycerols and/or by sequestering CGI-58 (196). In the activated state, phosphorylation of PLN1 results in the release of CGI-58, which can then activate ATGL (197). Therefore, further research is needed to evaluate how fasting affects phosphorylated PLN1 levels in the WAT of chickens. MGLL liberates the final fatty acid from the glycerol backbone of diacylglycerols; *MGLL* was not upregulated after fasting in any adipose tissue depot. Fasting increased *NPY* and *ACADL* mRNA and decreased *CGI-58* mRNA expression in the clavicular fat of chicks. ACADL is an enzyme that catalyzes the first step of the β-oxidation of long chain fatty acids in the mitochondria (198). The differential expression of these genes might indicate that the clavicular adipose tissue has a different metabolic response to fasting than subcutaneous and abdominal fat.

According to Nguyen et al. (2014), there was a greater sympathetic drive, triggered by food deprivation, to subcutaneous (inguinal) WAT than visceral (mesenteric) WAT depots, with increased sympathetic nervous drive only being observed in the subcutaneous (inguinal) WAT after the lipolytic stimulus of food deprivation in Siberian hamsters (199). In general,

sympathetic stimulation promotes lipolysis in WAT (200). Therefore, there might be a proportionally greater increase in lipolysis in the subcutaneous fat than the other two depots.

Similar to fasting, there is also a cascade of events during refeeding after fasting that may allow tissues such as adipose tissue to be reprogrammed at the metabolic level to maintain nutritional homeostasis. In the WAT of rats, 3 h of refeeding was sufficient to restore the mRNA expression of *PPARγ* and *SREBP1* to control levels after 8 h of fasting, however, this time did not allow for the recovery of *FAS* (189). In the present study, fasting increased plasma NEFA concentrations in chicks fed the HC and HP diets, and 1 h of refeeding restored the plasma NEFA concentrations to the fed state. However, 1 h of refeeding was not sufficient for restoring the fed-state levels of mRNA expression of some adipogenesis-associated factors, such as *PPARγ* and *SREBP1*.

Conclusions

In conclusion, reduced adipose tissue deposition in chicks fed a HP diet might be explained by decreased rates of adipogenesis and associated fatty acid incorporation into triacylglycerol synthesis. Conversely, chicks that consumed the HF diet had heavier fat pads and larger adipocytes, likely as a result of greater rates of adipocyte hypertrophy. In response to fasting, transcriptional regulation of adipogenesis-associated factors is induced, the net effect likely being an inhibition of preadipocyte differentiation and adipocyte hypertrophy, in a depot-specific manner. The lipolytic response to 3 h of fasting in chicks that consumed the HP diet also supports that the balance between adipogenesis/hypertrophy and lipid catabolism is shifted to favor a reduced rate of fat deposition. These results may provide implications for understanding how dietary macronutrients affect the development of different adipose tissue depots during the

early life period and mechanisms underlying depot-dependent differences in metabolism across species.

Table 3-1. Ingredient and chemical composition of experimental chick diets.

Ingredient (% as fed)	High-carbohydrate	High-protein	High-fat
Corn grain	60.37	43.61	31.28
Soybean meal	33.99	48.80	36.94
Soy hulls	0.00	0.00	17.27
Dicalcium phosphate	1.50	1.42	1.56
Limestone	1.21	1.14	0.89
Soybean oil	1.13	3.65	10.20
Vitamin/mineral premix[a]	1.00	1.00	1.00
Methionine 99 %	0.26	0.12	0.32
L-Lysine HCL 78 %	0.19	0.00	0.16
Sodium bicarbonate	0.15	0.15	0.15
L-Threonine	0.09	0.00	0.12
Coban 90[b]	0.05	0.05	0.05
Choline-Cl 60 %	0.05	0.05	0.05
Phytase-Ronozyme[c]	0.01	0.01	0.01
Kcal ME/kg	3,000	3,000	3,000
Crude protein (%)[d]	22	25	22
Crude fat (%)[d]	3.0	4.6	10.2
Crude fiber (%)	1.8	8.3	1.8

[a]Guaranteed analysis (per kg of premix): Manganese, 25.6 g; selenium, 120 mg; zinc, 30 g; Vitamin A, 4409, 171.076 IU; Vitamin D3, 1410,934.744 ICU; 13,227.513 IU; D-biotin, 88.183 mg.

[b]Coban 90 (Elanco Animal Health) contains 90 g of Monensin sodium per pound of premix and is included in the diet as a coccidiostat.

[c]DSM Nutritional Products, Ltd.

[d]Analyzed at Experiment Station Chemical Laboratories at University of Missouri

Table 3-2. Primers used for real time PCR.

Gene[1]	Primers sequence (5'-3'); Forward/Reverse	Accession No.
β-actin	GTCCACCGCAAATGCTTCTAA/TGCGCATTTATGGGTTTTGTT	NM_205518.1
PPARγ	CACTGCAGGAACAGAACAAAGAA/TCCACAGAGCGAAACTGACATC	NM_001001460.1
C/EBPα	CGCGGCAAATCCAAAAAG/GGCGCACGCGGTACTC	NM_001031459.1
C/EBPβ	GCCGCCCGCCTTTAAA/CCAAACAGTCCGCCTCGTAA	NM_205253.2
FABP4	CAGAAGTGGGATGGCAAAGAG/CCAGCAGGTTCCCATCCA	NM_204290.1
SREBP1	CATCCATCAACGACAAGATCGT/CTCAGGATCGCCGACTTGTT	NM_204126.1
KLF7	GATGCTGGTTTTCCTCACAGTTT/CCTCCTGTCCCAAAAGTGTTCA	XM_004942644.2
GATA2	CCACGAAGCAAGGCCAGAT/GGTAGCGGTTGCTCCACAGT	XM_015293080.1
AGPAT9	CCCATAGATGCGATCATTTTGA/CGTGAACTTGGCCAACCAT	NM_001031145.1
MGLL	GCAGACGAGCATAGACTCA/GGGAATAGCCTGGTTTACAA	XM_015293082.1
ATGL	GCCTCTGCGTAGGCCATGT/GCAGCCGGCGAAGGA	NM_001113291.1
CGI-58	CGCCCAGTGGTGAAAC/GCCTTTTTGCCCATCCATAA	NM_001278145.1
PLN1	GGAGGACGTGGCATGATGAC/GGCCCTTCCATTCTGCAA	NM_001127439.1
ACADL	GACATCGGCACTCGGAAGA/CCTGGTGCTCTCCCTGAAGA	NM_001006511.2
LPL	GACAGCTTGGCACAGTGCAA/CACCCATGGATCACCACAAA	NM_205282.1
NPY	CATGCAGGGCACCATGAG/CAGCGACAAGGCGAAAGTC	M87294.1
NPYR2	TGCCTACACCCGCATATGG/GTTCCCTGCCCCAGGACTA	NM_001031128.1

[1]Gene abbreviations: Peroxisome proliferator-activated receptor gamma: *PPARγ*; CCAAT/enhancer-binding protein alpha and beta: *C/EBPα* and *C/EBPβ*, respectively; Fatty acid binding protein 4: *FABP4*; Sterol regulatory element-binding transcription factor 1: *SREBP1*; Kruppel-Like Factor 7: *KLF7*; GATA binding protein 2: *GATA2*; 1-acylglycerol-3-phosphate O-acyltransferase 9: *AGPAT9*; Monoglyceride lipase: *MGLL*; Adipose triglyceride lipase: *ATGL*; Comparative gene identification-58: *CGI-58*; Perilipin 1: *PLN1*; Acyl-CoA dehydrogenase, long chain: *ACADL*; Lipoprotein lipase: *LPL*; Neuropeptide Y: *NPY*; *NPY* receptor 2: *NPYR2*.

Table 3-3. Adiopose tissue depot weights.

Effect[1]	Weights (g)	Weights (% BW)
Diet		
HC	0.33[a]	0.41[a]
HF	0.34[a]	0.42[a]
HP	0.23[b]	0.30[b]
SEM	0.017	0.021
P-value	0.0001	0.0002
Depot		
Subcutaneous	0.52[a]	0.65[a]
Clavicular	0.27[b]	0.34[b]
Abdominal	0.11[c]	0.14[c]
SEM	0.017	0.021
P-value	0.0001	0.0001
D × D	0.08	0.20

[1]Effects of diet (high-carbohydrate: HC; high-fat: HF; and high-protein: HP), adipose tissue depot (subcutaneous, clavicular and abdominal), and the interaction between diet (D) and depot (D) on adipose tissue weights and weights as a percentage of body weight. Values represent least squares means and pooled standard errors of the means with associated *P*-values for each effect (n = 10). Unique superscipts within an effect are significantly different at $P < 0.05$, Tukey's test.

Table 3-4. Adipocyte area and diameter in different adipose tissue depots.

Effect[1]	Area (µm)	Diameter (µm)
Diet		
HC	368.5	20.3[b]
HF	442.4	22.4[a]
HP	361.4	20.3[b]
SEM	25.1	0.6
P-value	0.056	0.02
Depot		
Subcutaneous	526.0[a]	24.6[a]
Clavicular	419.1[b]	22.0[b]
Abdominal	227.1[c]	16.4[c]
SEM	25.1	0.6
P-value	0.0001	0.0001
D × D	0.96	0.97

[1]Main effects of diet (high-carbohydrate: HC; high-fat: HF; and high-protein: HP), adipose tissue depot (subcutaneous, clavicular and abdominal), and the interaction between diet (D) and depot (D) on adipocyte area and diameter. Images were analyzed using NIS-Elements Advanced Research Software (Nikon). The density, diameter, and area of all adipocytes within the field of an image were measured. Adipocytes were treated as binary objects with the restriction that measurements must exceed 100 μm^2. Values represent least squares means and pooled standard errors of the means with associated *P*-values for each effect (HC: n = 7; HF: n = 6; HP: n = 7). Different superscipts within an effect are significantly different at $P < 0.05$, Tukey's test.

Table 3-5. Subcutaneous fat mRNA abundance at 4 days post-hatch.

Effect[1]	*AGPAT9*	*FABP4*	*C/EBPα*	*C/EBPβ*	*KLF7*	*PPARγ*	*MGLL*	*SREBP1*
Diet								
HC	0.44	1.06[a]	0.35	1.77	1.31	0.45	2.60	0.51
HF	0.36	1.00[ab]	0.32	1.40	1.32	0.50	2.21	0.43
HP	0.36	0.84[b]	0.32	1.33	1.19	0.39	2.31	0.41
SEM	0.04	0.06	0.02	0.18	0.09	0.04	0.27	0.04
P-value	0.23	0.02	0.37	0.21	0.66	0.27	0.55	0.10
Fed	0.57[a]	1.03	0.51[a]	1.00[b]	1.28[b]	0.73[a]	2.42	0.67[a]
Fasted	0.31[b]	0.96	0.16[c]	2.26[a]	1.59[a]	0.32[b]	2.54	0.39[b]
Refed	0.31[b]	0.91	0.33[b]	1.18[b]	0.97[c]	0.32[b]	2.18	0.33[b]
SEM	0.04	0.05	0.02	0.17	0.09	0.04	0.26	0.04
P-value	0.0001	0.35	0.0001	0.0001	0.0001	0.0001	0.57	0.0001
D × T	0.13	0.22	0.003	0.31	0.77	0.21	0.67	0.06

Table 3-5. Continued

Effect[1]	*GATA2*	*ATGL*	*PLN1*	*CGI-58*	*ACADL*	*NPY*	*NPYR2*	*LPL*
Diet								
HC	0.97	1.41	0.89	1.24	0.70	0.91	0.69	1.21
HF	1.05	1.42	0.77	0.70	0.56	0.64	0.66	1.41
HP	0.86	1.36	0.87	0.96	0.49	1.09	0.61	1.64
SEM	0.07	0.17	0.13	0.19	0.15	0.26	0.05	0.19
P-value	0.11	0.96	0.76	0.15	0.57	0.46	0.48	0.30
Treatment								
Fed	1.10	1.50	0.79	1.20	0.72	1.35	0.89[a]	1.55
Fasted	0.90	1.38	1.00	0.93	0.76	0.85	0.56[b]	1.25
Refed	0.89	1.31	0.74	0.77	0.28	0.44	0.50[b]	1.47
SEM	0.06	0.17	0.13	0.19	0.15	0.26	0.05	0.19
P-value	0.08	0.76	0.30	0.29	0.03	0.07	0.0001	0.50
D × T	0.26	0.76	0.56	0.51	0.14	0.76	0.03	0.37

[1]Values represent least squares means and pooled standard errors of the means with associated *P*-values for each effect. D × T: interaction between diet (D) and treatment (T). Different superscipts within an effect for each gene are significantly different at $P < 0.05$, Tukey's test. Abbreviations: 1-acylglycerol-3-phosphate O-acyltransferase 9: *AGPAT9*; Fatty acid binding protein 4: *FABP4*; CCAAT/enhancer-binding protein alpha and beta: *C/EBPα* and *C/EBPβ*, respectively; Krüppel-like factor 7: *KLF7*; Peroxisome proliferator-activated receptor gamma: *PPARγ*; Monoglyceride lipase: *MGLL*; Sterol regulatory element-binding transcription factor 1: *SREBP1*; GATA-binding protein 2: *GATA2*; Adipose triglyceride lipase:

ATGL; Perilipin 1: *PLN1*; Comparative gene identification-58: *CGI-58*; Acyl-CoA dehydrogenase, long chain: *ACADL*; Neuropeptide Y: *NPY*; NPY receptor 2: *NPYR2;* Lipoprotein lipase: *LPL*.

Table 3-6. Clavicular fat mRNA abundance at 4 days post-hatch.

Effect[1]	*AGPAT9*	*FABP4*	*C/EBPα*	*C/EBPβ*	*KLF7*	*PPARγ*	*MGLL*	*SREBP1*
Diet								
HC	0.62	0.90[a]	0.57	2.08	1.02	0.54	1.34[a]	0.69[a]
HF	0.48	1.09[a]	0.78	2.12	1.01	0.50	1.40[a]	0.60[ab]
HP	0.51	0.68[b]	0.50	1.30	1.09	0.44	0.98[b]	0.33[c]
SEM	0.09	0.06	0.15	0.33	0.07	0.05	0.12	0.07
P-value	0.34	0.0001	0.41	0.18	0.66	0.19	0.03	0.0008
Treatment								
Fed	0.77[a]	0.97[a]	0.84	1.12[b]	0.99[b]	0.78[a]	1.25	0.82[a]
Fasted	0.51[ab]	0.93[ab]	0.58	2.68[a]	1.33[a]	0.39[b]	1.13	0.41[b]
Refed	0.37[b]	0.76[b]	0.45	1.66[ab]	0.81[b]	0.33[b]	1.32	0.40[b]
SEM	0.08	0.06	0.15	0.32	0.07	0.05	0.12	0.07
P-value	0.006	0.04	0.19	0.007	0.0001	0.0001	0.51	0.0001
D × T	0.01	0.87	0.19	0.97	0.54	0.04	0.06	0.008

Table 3-6. Continued

Effect[1]	*GATA2*	*ATGL*	*PLN1*	*CGI-58*	*ACADL*	*NPY*	*NPYR2*	*LPL*
Diet								
HC	0.85	0.65	0.91[a]	0.59	1.58	1.02	0.56	1.33
HF	0.87	0.49	1.09[a]	0.56	1.54	1.01	0.51	1.33
HP	0.73	0.51	0.68[b]	0.50	0.94	1.09	0.44	0.98
SEM	0.05	0.08	0.06	0.08	0.21	0.07	0.05	0.12
P-value	0.12	0.34	0.0001	0.71	0.07	0.66	0.19	0.05
Treatment								
Fed	0.86	0.77[a]	0.98[a]	0.84[a]	1.03[b]	0.99[b]	0.79[a]	1.19
Fasted	0.78	0.51[ab]	0.93[ab]	0.37[b]	1.82[a]	1.33[a]	0.39[b]	1.13
Refed	0.81	0.37[b]	0.77[b]	0.45[b]	1.21[ab]	0.81[b]	0.33[b]	1.32
SEM	0.05	0.08	0.06	0.08	0.22	0.07	0.05	0.12
P-value	0.57	0.0058	0.04	0.0001	0.04	0.0001	0.0001	0.49
D × T	0.30	0.01	0.87	0.17	0.51	0.54	0.04	0.19

[1]Values represent least squares means and pooled standard errors of the means with associated *P*-values for each effect. D × T: interaction between diet (D) and treatment (T). Different superscipts within an effect for each gene are significantly different at $P < 0.05$, Tukey's test. Abbreviations: 1-acylglycerol-3-phosphate O-acyltransferase 9: *AGPAT9*; Fatty acid binding protein 4: *FABP4*; CCAAT/enhancer-binding protein alpha and beta: *C/EBPα* and *C/EBPβ*, respectively; Krüppel-like factor 7: *KLF7*; Peroxisome

proliferator-activated receptor gamma: *PPARγ*; Monoglyceride lipase: *MGLL*; Sterol regulatory element-binding transcription factor 1: *SREBP1*; GATA-binding protein 2: *GATA2*; Adipose triglyceride lipase: *ATGL*; Perilipin 1: *PLN1*; Comparative gene identification-58: *CGI-58*; Acyl-CoA dehydrogenase, long chain: *ACADL*; Neuropeptide Y: *NPY*; *NPY* receptor 2: *NPYR2*; Lipoprotein lipase: *LPL*.

Table 3-7. Abdominal fat mRNA abundance at 4 days post-hatch.

Effect[1]	*AGPAT9*	*FABP4*	*C/EBPα*	*C/EBPβ*	*KLF7*	*PPARγ*	*MGLL*	*SREBP1*
Diet								
HC	0.39	0.74	0.36	2.07^{a}	1.62^{ab}	0.38	4.27^{a}	0.43
HF	0.34	0.75	0.36	1.50^{ab}	1.63^{a}	0.41	3.33^{ab}	0.43
HP	0.34	0.71	0.40	1.10^{b}	1.33^{b}	0.42	2.86^{b}	0.38
SEM	0.03	0.05	0.03	0.17	0.08	0.03	0.38	0.03
P-value	0.43	0.90	0.56	0.0006	0.02	0.72	0.03	0.15
Treatment								
Fed	0.42^{a}	0.69	0.50^{a}	1.21^{b}	1.32^{b}	0.55^{a}	4.16^{a}	0.64^{a}
Fasted	0.35^{ab}	0.79	0.27^{c}	1.92^{a}	1.91^{a}	0.27^{c}	5.30^{a}	0.36^{b}
Refed	0.30^{b}	0.72	0.36^{b}	1.48^{ab}	1.32^{b}	0.41^{b}	1.03^{b}	0.26^{c}
SEM	0.03	0.05	0.02	0.16	0.08	0.03	0.38	0.03
P-value	0.02	0.30	0.0001	0.01	0.0001	0.0001	0.0001	0.0001
D × T	1.00	0.41	0.37	0.46	0.18	0.16	0.11	0.16

Table 3-7. Continued

Effect[1]	*GATA2*	*ATGL*	*PLN1*	*CGI-58*	*ACADL*	*NPY*	*NPYR2*	*LPL*
Diet								
HC	0.77	1.42	0.78	0.55	0.65^{ab}	0.73	0.30	1.51
HF	0.88	0.74	0.56	0.74	0.55^{b}	1.02	0.30	1.55
HP	0.67	0.50	0.68	0.58	0.86^{a}	1.03	0.31	1.60
SEM	0.07	0.41	0.13	0.07	0.09	0.14	0.04	0.21
P-value	0.14	0.25	0.47	0.18	0.04	0.24	1.00	0.86
Treatment								
Fed	0.80	0.60	0.58	0.63	0.67	1.49^{a}	0.33^{ab}	1.67
Fasted	0.78	0.66	0.74	0.57	0.72	0.84^{b}	0.36^{a}	1.52
Refed	0.74	1.41	0.71	0.67	0.67	0.44^{b}	0.21^{c}	1.56
SEM	0.08	0.41	0.12	0.07	0.09	0.15	0.04	0.22
P-value	0.83	0.29	0.63	0.68	0.89	0.0005	0.04	0.89
D × T	0.97	0.18	0.24	0.02	0.12	0.48	0.03	0.66

[1]Values represent least squares means and pooled standard errors of the means with associated *P*-values for each effect. D × T: interaction between diet (D) and treatment (T). Different superscipts within an effect for each gene are significantly different at $P < 0.05$, Tukey's test. Abbreviations: 1-acylglycerol-3-phosphate O-acyltransferase 9: *AGPAT9*; Fatty acid binding protein 4: *FABP4*; CCAAT/enhancer-binding protein alpha and beta: *C/EBPα* and *C/EBPβ*, respectively; Krüppel-like factor 7: *KLF7*; Peroxisome

proliferator-activated receptor gamma: *PPARγ*; Monoglyceride lipase: *MGLL*; Sterol regulatory element-binding transcription factor 1: *SREBP1*; GATA-binding protein 2: *GATA2*; Adipose triglyceride lipase: *ATGL*; Perilipin 1: *PLN1*; Comparative gene identification-58: *CGI-58*; Acyl-CoA dehydrogenase, long chain: *ACADL*; Neuropeptide Y: *NPY*; *NPY* receptor 2: *NPYR2*; Lipoprotein lipase: *LPL*.

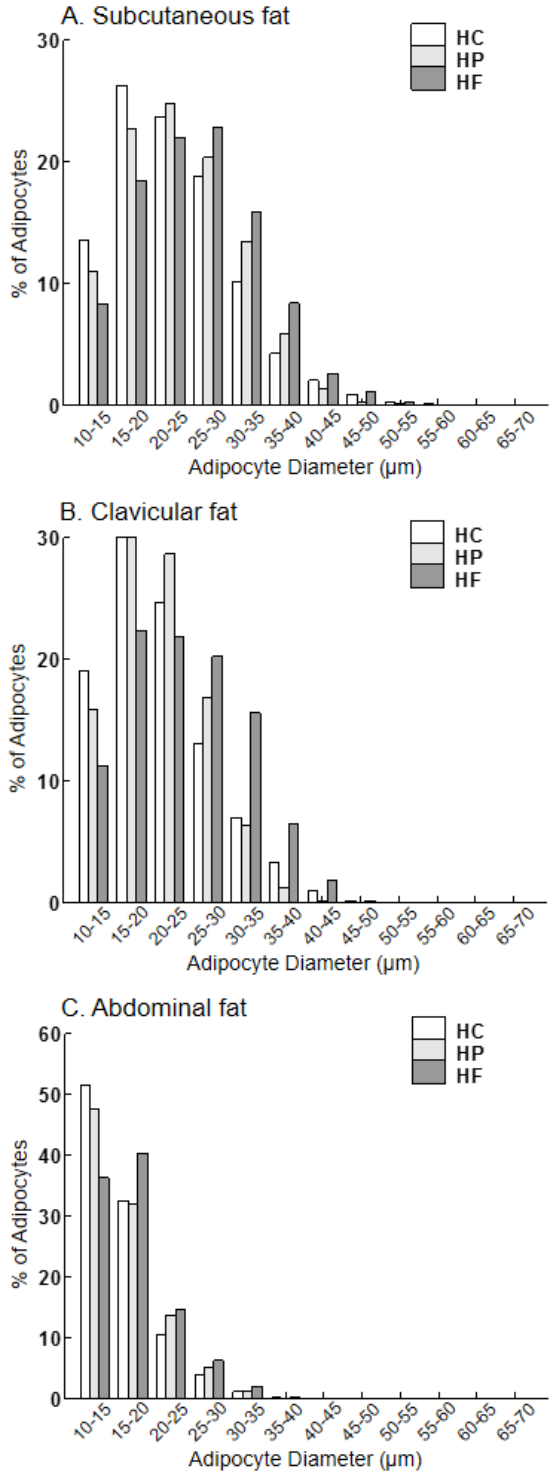

Figure 3-1. Adipocyte size distribution in the three adipose tissue depots of chicks from different dietary groups. (A) subcutaneous, (B) clavicular, and (C) abdominal adipose tissues were sampled at day 4 post-hatch in chicks fed a high-carbohydrate (HC), high-protein (HP) or high-fat (HF) diet. Images were

captured with a Nikon Eclipse 80i microscope and DS-Ri1 color camera and analyzed using NIS-Elements Advanced Research Software (Nikon). The density, diameter, and area of all adipocytes within the field of an image were measured under 20x magnification. The threshold method was used to count adipocytes. n = 7 (HC), 6 (HF) and 7 (HP).

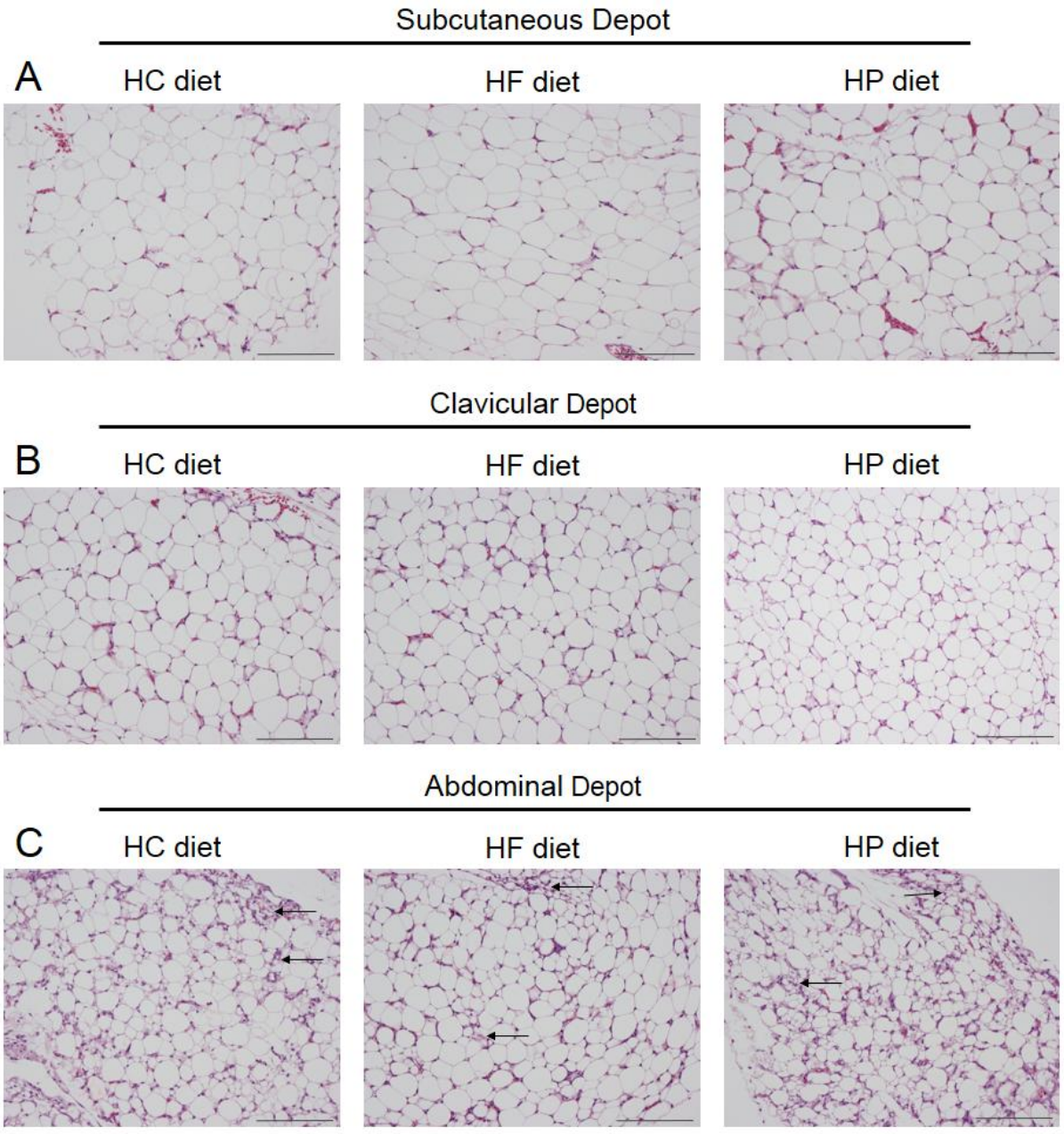

Figure 3-2. Adipose tissue histology at day 4 post-hatch in different adipose tissue depots. Scale bar = 100 µm. Representative images of hematoxylin and eosin-stained sections in the (A) subcutaneous, (B) clavicular, and (C) abdominal depots from n = 7 (high-carbohydrate diet; HC), 6 (high-fat diet; HF) and 7 (high-protein diet; HP). Images were captured with a Nikon Eclipse 80i microscope and DS-Ri1 color camera.

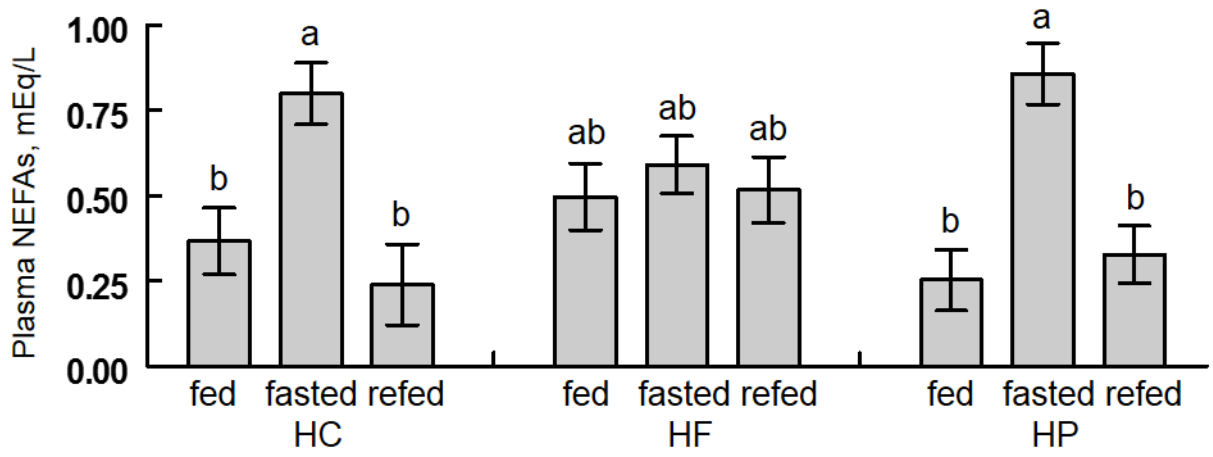

Figure 3-3. Interaction of diet and treatment on plasma non-esterified fatty acid concentrations. At day 4 post-hatch, chicks fed one of three diets (HC: high-carbohydrate; HF: high-fat; HP: high-protein) were either continuously fed (fed), fasted for 3 h (fasted) or fasted and refed for 1 hour (refed) with n =10 per group. Values of non-esterified fatty acid concentrations (NEFAs) represent least squares means ± SEM. Different letters indicate a significant difference at $P < 0.05$; Tukey's test.

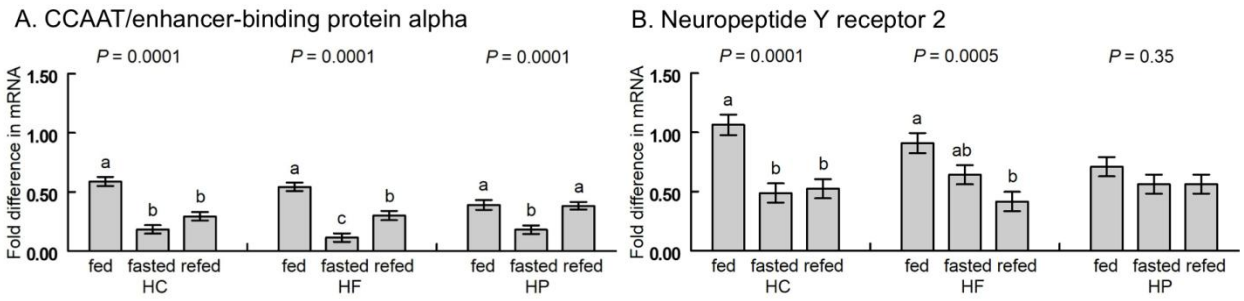

Figure 3-4. Interactions of diet and treatment on mRNA abundance in subcutaneous fat. Chicks fed one of three diets (HC: high-carbohydrate; HF: high-fat; HP: high-protein) were either continuously fed (fed), fasted for 3 h (fasted) or fasted and refed for 1 hour (refed) with n =10 per group. Values represent least squares means ± SEM. The *P*-values for the main effect of treatment are displayed above the bars for each dietary group. Different letters within a dietary group indicate a significant difference at $P < 0.05$; Tukey's test.

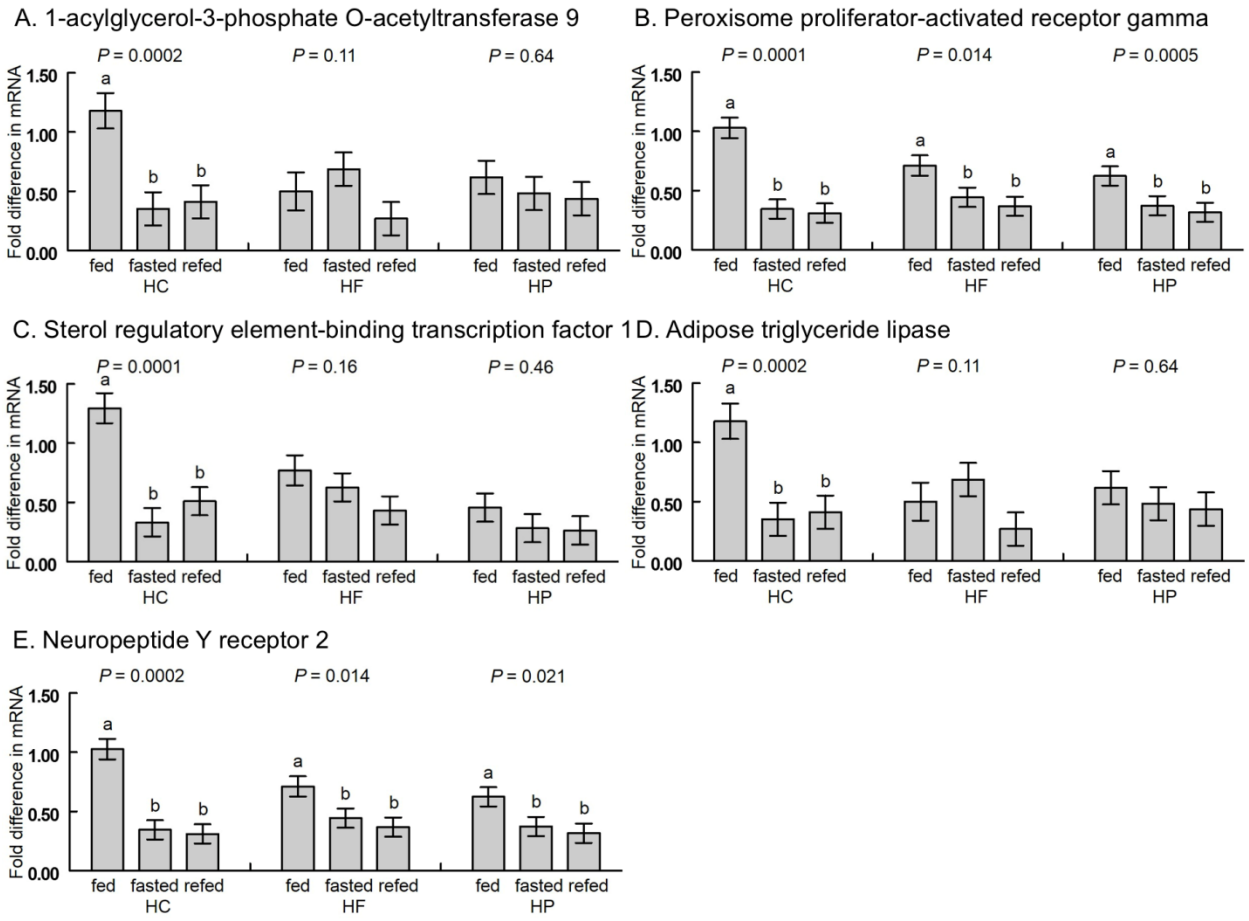

Figure 3-5. Interactions of diet and feeding treatment on mRNA abundance in clavicular fat. Chicks fed one of three diets (HC: high-carbohydrate; HF: high-fat; HP: high-protein) were either continuously fed (fed), fasted for 3 h (fasted) or fasted and refed for 1 hour (refed) with n =10 per group. Values represent least squares means ± SEM. The *P*-values for the main effect of treatment are displayed above the bars for each dietary group. Different letters within a dietary group indicate a significant difference at $P < 0.05$; Tukey's test.

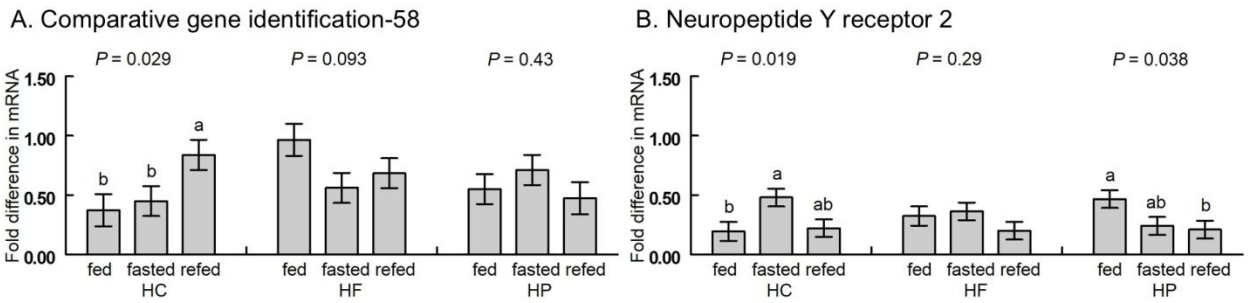

Figure 3-6. Interactions of diet and feeding treatment on mRNA abundance in abdominal fat. Chicks fed one of three diets (HC: high-carbohydrate; HF: high-fat; HP: high-protein) were either continuously fed (fed), fasted for 3 h (fasted) or fasted and refed for 1 hour (refed) with n =10 chicks per group. Values represent least squares means ± SEM. The P-values for the main effect of treatment are displayed above the bars for each dietary group. Different letters within a dietary group indicate a significant difference at $P < 0.05$; Tukey's test.

Chapter 4: A high fat diet enhances the sensitivity of chick adipose tissue to the effects of centrally-injected neuropeptide Y on gene expression of adipogenesis-associated factors

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Abstract

The purpose of this study was to determine how dietary macronutrient composition and exogenous neuropeptide Y (NPY) affect lipid metabolism-associated factor mRNA in chick adipose tissue. Chicks were fed one of three isocaloric (3,000 kcal metabolizable energy (ME)/kg) diets after hatch: high carbohydrate (HC; control), high fat (HF; 30% of ME from soybean oil) or high protein (HP; 25% crude protein). On day 4 post-hatch, vehicle or 0.2 nmol of NPY was injected intracerebroventricularly and abdominal and subcutaneous fat depots collected 1 hour later. In abdominal fat, mRNA abundance of peroxisome proliferator-activated receptor γ (PPARγ) and fatty acid binding protein 4 (FABP4) increased after NPY injection in HF diet-fed chicks. NPY injection decreased expression of PPARγ and sterol regulatory element-binding transcription factor 1 (SREBP1) in the subcutaneous fat of HC diet-fed chicks, whereas SREBP1 expression was increased in the subcutaneous fat of HF diet-fed chicks after NPY injection.

An acutely increased central concentration of NPY in chicks affects adipose tissue physiology in a depot- and diet-dependent manner. The chick may serve as a model to understand the relationship between diet and the brain-fat axis' role in maintaining whole body energy homeostasis, as well as to understand metabolic distinctions among fat depots.

Key words: adipose tissue, chick, dietary macronutrient, neuropeptide Y, peroxisome proliferator-activated receptor γ

Introduction

Dietary macronutrient composition affects adipose tissue physiology in mammalian and avian species. Consumption of a high fat (HF) diet was associated with increased adipocyte volume in rats (201). Effects on adipose tissue are depot-dependent in that visceral fat expands predominantly by hypertrophy while hyperplasia contributes more to subcutaneous fat development in mice fed a HF diet (172). Consumption of a high protein (HP) diet was associated with decreased gene expression of adipogenesis-associated transcription factors in the adipose tissue of 4 day-old chicks, and differences in adipocyte morphology and plasma non-esterified fatty acids (NEFAs) from chicks that consumed high carbohydrate (HC) or HF diets (202).

Adipose tissue development is also influenced by appetite-regulating peptides, such as neuropeptide Y (NPY), a 36 amino acid neurotransmitter that is involved in the regulation of energy balance. Obesity is associated with elevated hypothalamic NPY while induction of NPY expression in the hypothalamus results in obesity (203-205). NPY is a potent orexigenic factor that increases food intake in both mammalian (206) and avian species (89), and the magnitude of response in chicks is affected by dietary macronutrient composition (173, 207). Besides its orexigenic effects, central NPY injection is associated with enhanced lipid storage in white adipose tissue (WAT) in rats (208), and our group demonstrated that NPY promotes adipogenesis during the early and later stages of chicken preadipocyte differentiation (95, 96).

Thus, based on previous studies showing a role for NPY in regulating both appetite and adiposity in chickens and effects of dietary macronutrient composition on the appetite response to NPY and adipose tissue development in chicks, this study was designed to determine how dietary macronutrient composition and centrally-administered NPY affect gene expression of adipogenesis-associated factors in chicks.

Materials and methods

Experimental animals

Cobb-500 broiler chicks were obtained from a commercial hatchery on the day of hatch, group caged and 1 day later were caged individually in a room at 30 ± 1 ℃ and 50 ± 5 % relative humidity. Chicks were handled daily to adapt to handling and minimize stress, with ad libitum access to diet and tap water. From day of hatch, chicks were assigned to receive one of the three diets, which were formulated to be isocaloric (3,000 kcal/kg) as shown in Table 4-1 (207). The HC diet was formulated to meet the minimum requirements defined for the starter phase of commercial broilers (Cobb-Vantress), the HP diet contained 25 % crude protein, and the HF diet was formulated to have 30 % of the metabolizable energy derived from soybean oil. Crude protein and fat values were experimentally verified for all diets (Table 4-1). Experimental procedures were performed according to the National Research Council Publication, Guide for Care and Use of Laboratory Animals and were approved by the Virginia Tech Institutional Animal Care and Use Committee.

Intracerebroventricular (ICV) injection procedure

At day 4 post-hatch, the chicks were assigned to treatment groups (three diet groups receiving injection of vehicle or 0.2 nmol NPY; 6 total groups) using a randomized complete block design

with body weight as the blocking factor. Chicks were injected using a method adapted from Davis et al. (1979) that does not appear to induce physiological stress (209, 210). The dose of chicken NPY was the same as that used in our previous study that examined the effects of diet and central NPY on dietary preference and hypothalamic gene expression (207). Chicken NPY (YPSKPDSPGEDAPAEDMARYYSALRHYINLITRQRY, AnaSpec, San Jose, CA, USA) was dissolved in artificial cerebrospinal fluid (211) as a vehicle for a total injection volume of 5 µl with 0.06 % Evans Blue dye to facilitate injection site localization. After data collection, chicks were decapitated and the head sectioned along the frontal plane to determine the site of injection. Any chick without dye present in the lateral ventricle system was eliminated from analysis. Sex of chicks was determined visually by dissection. The number of chicks for each experiment are depicted in the figure legends.

Total RNA extraction, reverse transcription, and real time PCR

At one hour post-injection, chicks were deeply anesthetized with sodium pentobarbital via cardiopuncture. Adipose tissue depots, including abdominal and subcutaneous, were collected and processed for total RNA isolation as described (202). Briefly, samples were homogenized in 1 mL Isol RNA Lysis reagent (5-Prime, Gaithersburg, MD, USA) using 5 mm stainless steel beads (Qiagen, Valencia, CA, USA) and a Tissue Lyser II (Qiagen). Total RNA was separated following the manufacturer's instructions (5-Prime) and following the step of addition to 70 % molecular-grade ethanol, samples were transferred to spin columns and further purified using the RNeasy Mini Kit (Qiagen). First-strand cDNA was synthesized from 200 ng total RNA with a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Carlsbad, CA, USA). Primers for real time PCR were designed with Primer Express 3.0 (Applied Biosystems) and validated for amplification efficiency before use (Table 4-2). Real time PCR was performed in

duplicate in 10 µl reactions that contained 5 µl Fast SYBR Green Master Mix (Applied Biosystems), 0.25 µl of 5 µM forward primer, 0.25 µl of 5 µM reverse primer, 1.5 µl of nuclease free water and 3 µl of 10-fold diluted cDNA using a 7500 Fast Real-Time PCR System (Applied Biosystems). A dissociation step was performed at the end of each PCR reaction to ensure amplicon specificity.

Statistical analysis

The real time PCR data were analyzed using the ΔΔCT method, where $\Delta CT = CT_{\text{target gene}} - CT_{\text{actin}}$, and $\Delta\Delta CT = \Delta CT_{\text{target sample}} - \Delta CT_{\text{calibrator}}$ (175). The average of subcutaneous fat from chicks fed the HC diet that also received the vehicle treatment was used as the calibrator sample. The fold difference (relative quantity; RQ) was calculated as $2^{-\Delta\Delta CT}$. Analysis of variance was performed with RQ values using the Fit Model platform of JMP Pro11 (SAS Institute, Cary, NC). The statistical model included the main effects of diet, NPY treatment and the interaction between them, within an adipose tissue depot. Sex was excluded as initial tests revealed that no effect involving sex was significant. Significant dietary effects were separated using Tukey's test and significant interactions separated with the Slice function of JMP and effects sliced within dietary group for each gene. When Slices were significant, a secondary ANOVA was performed with the model including the main effect of treatment within the diet and Tukey's test was used to separate the means for significant treatment effects. Significance was assigned at $P < 0.05$.

Data were transformed (212) as follows to reduce heterogeneity of variance, where x is the relative quantity value: 1/x: neuropeptide Y (NPY) (both depots), NPY receptor 2 (NPYR2) (both depots), CCAAT/enhancer-binding protein beta (C/EBPβ) (both depots), thioredoxin-dependent peroxidase 2 (TPX2) (abdominal), adipose triglyceride lipase (ATGL) (abdominal), comparative gene identification-58 (CGI58) (abdominal), GATA Binding Protein 2 (GATA2)

(abdominal), Ki67 (subcutaneous), lipoprotein lipase (LPL) (subcutaneous), 1-acylglycerol-3-phosphate O-acyltransferase 9 (AGPAT9) (subcutaneous), and fatty acid synthase (FASN) (subcutaneous); Square root (x) + square root (x+1): LPL (abdominal), acyl-CoA dehydrogenase, long chain (ACADL) (abdominal), and monoglyceride lipase (MGLL) (subcutaneous); Ln(x+1): perilipin 1 (PLN1) (both depots), MGLL (abdominal), ACADL (subcutaneous), CGI58 (subcutaneous), and GATA2 (subcutaneous); Log10(x): AGPAT9 (abdominal) and ATGL (subcutaneous); Log10(x+1): CCAAT/enhancer-binding protein alpha (C/EBPα) (both depots), sterol regulatory element-binding transcription factor 1 (SREBP1) (both depots), and FASN (abdominal); Square root (x+1): Kruppel-Like Factor (KLF7) (both depots) and fatty acid binding protein 4 (FABP4) (both depots). Ln(x): peroxisome proliferator-activated receptor gamma (PPARγ) (both depots). Values in results, tables, and figures reflect untransformed data.

Results

Gene expression results are summarized in Tables 4-3 and 4-4 (abdominal fat) and 4-5 and 4-6 (subcutaneous fat). There was no main effect of NPY treatment in either fat depot for any of the genes that were measured. There was a main effect of diet for every gene that was measured in abdominal and subcutaneous fat, except for C/EBPβ in subcutaneous fat. In addition, there were interactions of diet and NPY treatment on PPARγ and FABP4 mRNA in abdominal fat, and PPARγ and SREBP1 mRNA in subcutaneous fat.

Main effects of diet

In abdominal fat, C/EBPβ, PPARγ, SREBP1, FABP4, NPY and KLF7 mRNAs were greater in HC than HF or HP diet-fed chicks (Tables 4-3 and 4-4). Expression of MGLL and NPYR2 was greater in HF than HP diet-fed chicks. GATA2 mRNA was greater in HC than HF diet-fed

chicks in abdominal fat. The mRNA abundance of Ki67, topoisomerase II alpha (TOP2A), and TPX2 was greater in HC and HF than HP diet-fed chicks. Expression of PLN1, CGI-58, FASN, ACADL and AGPAT9 was greater in HF than HC or HP diet-fed chicks. Expression of C/EBPα was greatest in HC, intermediate in HP, and lowest in HF diet-fed chicks. LPL mRNA was greatest in HF, intermediate in HC, and lowest in HP diet-fed chicks. Adipose triglyceride lipase (ATGL) mRNA was greater in HC and HF than HP diet groups in the abdominal fat (Table 4-3).

In subcutaneous fat, expression of C/EBPα, PPARγ, FABP4, NPY, Ki67, and TPX2 were greater in HC than HF or HP diet-fed chicks (Tables 4-5 and 4-6). There was greater expression of NPYR2 in chicks fed the HC and HF diets than in chicks fed the HP diet. SREBP1 mRNA was greatest in HC, intermediate in HF, and lowest in HP diet-fed chicks. The expression of TOP2A was greater in HC than HP diet-fed chicks. LPL, ATGL, and AGPAT9 were greater in HF than HP diet-fed chicks. MGLL, PLN1, CGI-58, FASN and ACADL mRNAs were greater in HF than HC or HP diet-fed chicks. KLF7 and GATA2 mRNA quantities were greater in HC and HP than HF diet-fed chicks. NPYR2 mRNA was greater in HC and HF than HP diet-fed chicks (Table 4-6).

Diet by treatment interactions

In the abdominal fat of HF diet-fed chicks, PPARγ (Figure 4-1A) and FABP4 (Figure 4-1B) mRNAs were greater in the NPY than vehicle-injected group. PPARγ and SREBP1 mRNAs were greater in the subcutaneous fat of the HF diet-fed chicks after NPY injection, whereas PPARγ (Figure 4-2A) and SREBP1 (Figure 4-2B) decreased after NPY injection in the subcutaneous fat of chicks fed the HC diet.

Discussion

In a previous study that utilized the same diets as the present experiment, consumption of the HP diet was associated with decreased gene expression of adipogenesis-associated transcription factors in the adipose tissue of 4 day-old chicks (202). PPARγ and C/EBPα are key transcription factors that coordinately regulate adipocyte differentiation (59, 213). Subsequent to induction of C/EBP_{β} expression during adipocyte differentiation, PPARγ and C/EBPα are up-regulated (214) and SREBP1 enhances adipocyte differentiation in mammals (215) and chickens (214). There was decreased expression of PPARγ, C/EBPα, and SREBP1 in the abdominal and subcutaneous fat of chicks fed the HF and HP diets, compared with chicks fed the HC diet, which may indicate that there is reduced adipocyte differentiation in chicks fed the HF and HP diets. In the abdominal but not subcutaneous fat, there was less C/EBP_{β} mRNA in chicks fed the HF and HP diets compared with chicks fed the HC diet. As one of the direct inducers of PPARγ and C/EBPα expression (216), the downregulation of C/EBP_{β} mRNA may explain the decreased expression of PPARγ and C/EBPα in the abdominal fat.

KLF7 is more highly expressed in chicken preadipocytes than in mature adipocytes, and promotes preadipocyte proliferation (71). GATA2 has been described as a preadipocyte marker, as forced expression of GATA2 inhibited adipocyte differentiation from preadipocytes of the 3T3-F442A mouse cell line (217). In the abdominal fat, expression of GATA2 was greater in chicks fed the HC diet than in chicks fed the HF diet. In contrast, in the subcutaneous fat, HF diet feeding decreased the expression of these two preadipocyte markers, as compared with consumption of the HC and HP diets. Thus, there might be increased preadipocyte proliferation in the abdominal fat of chicks fed the HF and HP diets, as compared with chicks fed the HC diet.

Ki67, TOP2A and TPX2 proteins are proliferation markers (218, 219). In the abdominal fat of chicks fed the HP diet, there was relatively less expression of these three markers compared with chicks fed the HC and HF diets. However, in the subcutaneous fat, the expression of Ki67 and TPX2 was lower in chicks fed the HF and HP diets compared with chicks fed the HC diet, and TOP2A expression was decreased in chicks fed the HP diet compared with chicks fed the HC diet. Thus, there might be less preadipocyte proliferation in the abdominal fat of chicks fed the HP diet than in chicks fed the HC and HF diets, and in the subcutaneous fat of chicks fed the HF and HP diets compared with chicks fed the HC diet.

There was also relatively less FABP4 mRNA in the abdominal and subcutaneous fat of chicks that consumed the HF and HP diets. During adipogenesis, FABP4 plays an important role in triacylglycerol accumulation (220). It is a marker for adipocyte differentiation since its expression is increased during preadipocyte differentiation in both mammals (221) and chickens (114). Similar to PPARγ, C/EBPα and SREBP1, the decreased expression of FABP4 in the abdominal fat of chicks fed the HF and HP diets, compared with chicks fed the HC diet, may also suggest that there is reduced adipocyte differentiation in these two adipose tissue depots in chicks fed those diets. Since the expression of FABP4 is regulated by transcription factors such as PPARγ and C/EBPα (222), the downregulation of PPARγ and C/EBPα may also explain the decreased expression of FABP4 in the present study.

In contrast to the adipogenic markers, transcription factors, proliferation and preadipocyte markers, quantities of factors and enzymes associated with lipolysis and fatty acid oxidation were greater in both adipose tissue depots of chicks that consumed the HF diet. The ACADL is an enzyme that catalyzes the first step of the β-oxidation of long chain fatty acids in the mitochondria (198). Lipoprotein lipase activity declined during chicken adipocyte differentiation

(84), therefore, decreased LPL mRNA in the abdominal fat of chicks fed the HC and HP diets may indicate increased adipocyte differentiation in the abdominal fat of chicks that ate the HC and HP diets. In addition, LPL catalyzes the hydrolysis of triacylglycerol in very low density lipoproteins on endothelial cells lining the adipose tissue, a rate-limiting step in fat accretion in chickens (31). This also supports that chicks fed the HF diet have larger adipocytes.

In our previous study, chicks fed the HP diet had reduced adipose tissue weights compared with chicks fed the HC or HF diet (202). In the present study, the expression of most of the adipogenesis-associated factors in chicks fed the HP diet was lower than in chicks fed the HC diet, which supports the observation that adipose tissue depots weighed less in chicks fed the HP diet than in chicks fed the HC diet. In the present study, adipogenesis-associated factors were expressed similarly between HF and HP diet groups. Although chicks ate less of the HF diet than the other two diets (181), adipocytes were larger in chicks that consumed the HF diet (202), which might be explained by their increased sensitivity to NPY.

Two neuronal populations within the arcuate nucleus of the hypothalamus, proopiomelanocortin-expressing neurons and NPY/Agouti-related peptide (AgRP)-expressing neurons, have opposing effects on energy metabolism (223). The activation of NPY/AgRP neurons induces an anabolic state, for example, by reducing sympathetic outflow toward WAT to decrease lipolysis (200, 224). A recent study using Siberian hamsters showed that there were more neurons involved in innervating the inguinal (subcutaneous) fat compared with the mesenteric (visceral) fat pad, and interestingly, a greater sympathetic drive was observed in the inguinal WAT than visceral WAT under conditions of food deprivation (199). NPY is co-released with norepinephrine as a sympathetic nerve neurotransmitter, therefore, increased NPY expression might also indicate

increased sympathetic outflow toward peripheral organs (225). However, whether there is differential sympathetic innervation of the depots warrants further study.

In mammals, NPY, via NPYR2, enhances the proliferation of preadipocytes and differentiation into adipocytes, resulting in abdominal obesity (91). Knockout of NPYR2 decreased body weight gain and reduced adiposity in mice (92). Our group was the first to demonstrate that NPY also accentuates adipogenesis in avian species, using chickens as a model (95, 96). In the present study, there was less NPY mRNA in the subcutaneous and abdominal fat of chicks fed the HF and HP diets, compared with chicks fed the HP diet. Decreased NPY mRNA in these two adipose tissue depots suggests that there was reduced lipid deposition, which supports that there might be decreased adipogenesis in the two adipose tissue depots of chicks fed the HF and HP diets, also supported by the decreased expression of adipogenesis-associated factors.

There were significant two-way interactions of diet and NPY treatment on some adipogenesis-associated factors. In both fat depots, central NPY injection increased expression of adipogenesis-associated factors in chicks that consumed the HF diet, suggesting that consuming more fat makes chicks more sensitive to the adipogenesis-promoting effects of NPY. Similarly, chicks fed a HF diet were more sensitive to the effects of central NPY on increasing food intake (173). According to our *in vitro* study, chicken preadipocytes treated with exogenous NPY expressed less PPARγ on day 4 and 6, and decreased SREBP1 mRNA on day 4, post-induction of differentiation (95). In addition, the expression of PPARγ and FABP4 was up-regulated during the early stages of adipogenesis in cultured chicken preadipocytes after the addition of oleate (84). Interestingly, PPARγ and SREBP1 expression decreased in the subcutaneous fat of chicks fed the HC diet after ICV NPY injection. Thus, one may speculate that feeding the HF

diet may have sensitized precursor cells in subcutaneous fat to the adipogenesis-promoting effects of NPY.

Among the enzymes involved in triacylglycerol catabolism, ATGL and hormone sensitive lipase (HSL) are the major enzymes that account for more than 95% of the hydrolase activity in murine WAT (195). However, no orthologue of HSL has been identified in the chicken genome (45), indicating an even more important role of ATGL in lipolysis in chickens. Furthermore, MGLL liberates the final fatty acid from the glycerol backbone of diacylglycerols. Our previous study demonstrated that dietary macronutrient composition did not directly affect plasma non-esterified fatty acid (NEFA) concentrations, although HF diet-fed chicks were more resistant to a 3 hour fast-induced elevation in plasma NEFAs (202). In the present study, there was greater expression of ATGL and MGLL in the adipose tissue of chicks that consumed the HF diet, however it is unclear how expression of these enzymes is regulated at the protein level. Under basal conditions, PLN1 may suppress lipolysis by blocking the binding of lipase to triacylglycerols and/or by sequestering CGI58 (196). When activated, phosphorylation of PLN1 leads to the release of CGI58, which can then activate ATGL (197). Because HF diet-fed chicks were more resistant to fasting-induced rises in plasma NEFAs and adipocytes were larger (and adipose depots heavier) in the HF diet group (202), rates of lipolysis were likely similar with greater rates of adipogenesis in the adipose tissue of chicks consuming the HF diet.

Conclusions

In summary, for most genes evaluated in this study, expression was greater in either HC or HF diet- than HP diet-fed chicks, results suggesting that there is greater cellular proliferation and rates of adipogenesis in the adipose tissue of chicks that consumed the HC diet, and greater lipid remodeling and metabolism in the adipose tissue of chicks that consumed the HF diet. A number

of adipogenesis-associated factors were affected by central NPY injection in a diet and adipose depot-specific manner. These results may provide insight on adipose deposition in obese individuals. We hypothesize that there is positive feedback generated by increased distribution/signaling of NPY in the periphery, leading to enhanced adipogenesis and body fat accumulation in concert with increased hunger. Thus, dietary fat may lead to a more sensitized NPY system in the body although mechanisms are still unclear. The chicken may serve as a model to understand the relationship between diet and the brain-fat axis' role in maintaining whole body energy homeostasis.

Table 4-1. Ingredient and chemical composition of experimental diets.

Ingredient (g/kg)[a]	High carbohydrate	High protein	High fat
Corn grain	603.7	436.1	312.8
Soybean meal	339.9	488	369.4
Soy hulls	0	0	172.7
Dicalcium phosphate	15	14.2	15.6
Limestone	12.1	11.4	8.9
Soybean oil	11.3	36.5	102
Vitamin/mineral premix[b]	10	10	10
Methionine 99%	2.6	1.2	3.2
L-Lysine HCl 78%	1.9	0	1.6
Sodium bicarbonate	1.5	1.5	1.5
L-Threonine	0.9	0	1.2
Coban 90[c]	0.5	0.5	0.5
Choline-Cl 60%	0.5	0.5	0.5
Phytase-Ronozyme-10000[d]	0.1	0.1	0.1
Kcal ME/kg	3,000	3,000	3,000
Crude protein[e]	22%	25%	22%
Crude fat[e]	3.0%	4.6%	10.2%

[a]Diets were formulated to meet or exceed minimum recommended specifications for Cobb-500 broilers during the starter phase (Cobb-Vantress).

[b]Guaranteed analysis (per kg of premix): Manganese, 25.6 g; selenium, 120 mg; zinc, 30 g; Vitamin A, 4409, 171.076 IU; Vitamin D3, 1,410,934.744 ICU; 13,227.513 IU; d-biotin, 88.183 mg.

[c]Coban 90 (Elanco Animal Health) contains 90 g of Monensin sodium per pound of premix and is included in the diet as a coccidiostat.

[d]DSM Nutritional Products, Ltd.

[e]Analyzed at Experiment Station Chemical Laboratories at University of Missouri

Table 4-2. Primers used for real time PCR.

Gene	Sequence (5'-3')	Accession No.	Amplicon Size
β-Actin	F: GTCCACCGCAAATGCTTCTAA	NM_205518.1	78
	R: TGCGCATTTATGGGTTTTGTT		
KLF7	F: GATGCTGGTTTTCCTCACAGTTT	XM_004942644.2	69
	R: CCTCCTGTCCCAAAAGTGTTCA		
GATA2	F: CCACGAAGCAAGGCCAGAT	XM_015293080.1	66/99
	R: GGTAGCGGTTGCTCCACAGT		
C/EBPα	F: CGCGGCAAATCCAAAAAG	NM_001031459.1	55
	R: GGCGCACGCGGTACTC		
C/EBPβ	F: GCCGCCCGCCTTTAAA	NM_205253.2	59
	R: CCAAACAGTCCGCCTCGTAA		
PPARγ	F: CACTGCAGGAACAGAACAAAGAA	NM_001001460.1	66
	R:TCCACAGAGCGAACTGACATC		
SREBP1	F: CATCCATCAACGACAAGATCGT	NM_204126.1	82
	R: CTCAGGATCGCCGACTTGTT		
FABP4	F: CAGAAGTGGGATGGCAAAGAG	NM_204290.1	61
	R: CCAGCAGGTTCCCATCCA		
NPY	F: CATGCAGGGCACCATGAG	M87294	55
	R: CAGCGACAAGGCGAAAGTC		
NPYR2	F: TGCCTACACCCGCATATGG	NM_001031128.1	58
	R:GTTCCCTGCCCCAGGACTA		
Ki67	F: AAAAACCTGATTCCTGAACAATCTG	XM_004942360.1	104
	R: GACCTAGAGCTATCAGGCTGTGAAG		
TOP2A	F: GCACAGCTGGCGGAAGTAAT	NM_204791.1	63
	R: TGCAGTGACCCGAGGAACA		
TPX2	F: TGGAGGGTGGGCCAATC	NM_204437.1	57
	R: TTGGCTGTGTGAGTTCCTTCAC		
LPL	F: GACAGCTTGGCACAGTGCAA	NM_205282.1	62
	R: CACCCATGGATCACCACAAA		
MGLL	F: GCAGACGAGCATAGACTCA	XM_015293082.1	55
	R: GGGAATAGCCTGGTTTACAA		
ACADL	F: GACATCGGCACTCGGAAGA	NM_001006511.2	60
	R: CCTGGTGCTCTCCCTGAAGA		
AGPAT9	F: CCCATAGATGCGATCATTTTGA	NM_001031145.1	61
	R: CGTGAACTTGGCCAACCAT		
ATGL	F: GCCTCTGCGTAGGCCATGT	NM_001113291.1	60
	R: GCAGCCGGCGAAGGA		
PLN1	F: GGAGGACGTGGCATGATGAC	NM_001127439.1	57
	R: GGCCCTTCCATTCTGCAA		
CGI58	F: CGCCCAGTGGTGAAAC	NM_001278145.1	60
	R: GCCTTTTTGCCCATCCATAA		
FASN	F: CCATTGCACCAGCACTACTCA	NM_205155.2	59
	R: ACGAGGCTTAGGGTGTGGAA		

[1] Primers were designed with Primer Express 3.0 (Applied Biosystems). Abbreviations: Krüppel-like factor 7 (KLF7), GATA-binding protein 2 (GATA 2), CCAAT/enhancer-binding protein alpha (C/EBPα), CCAAT/enhancer-binding protein beta (C/EBPβ), Peroxisome proliferator-activated receptor gamma (PPARγ), Sterol regulatory element-binding transcription factor 1 (SREBP1), Fatty acid binding protein 4 (FABP4), Neuropeptide Y (NPY), Neuropeptide Y receptor 2 (NPYR2), Ki67, Topoisomerase II alpha (TOP2A), Targeting protein for Xklp2 (TPX2), Lipoprotein lipase (LPL), Monoglyceride lipase (MGLL), Acyl-CoA dehydrogenase long chain (ACADL), 1-acylgylcerol-3-phosphate O-acetyltransferase 9 (AGPAT9), Adipose triglyceride lipase (ATGL), Perilipin-1 (PLN1), Comparative gene identification-58 (CGI58), and Fatty acid synthase (FASN)

Table 4-3. Effects of diet and treatment on abdominal fat mRNA abundance of transcription factors and lipid metabolic enzymes.

Effect[1]	C/EBPα	C/EBPβ	PPARγ	SREBP1	LPL	ATGL	PLN1	CGI58	MGLL	FASN
Diet										
HC	1.10^{a}	1.10^{a}	1.01^{a}	1.08^{a}	0.95^{b}	0.94^{a}	0.95^{b}	0.99^{b}	1.10^{ab}	1.12^{b}
HF	0.25^{c}	0.38^{b}	0.25^{b}	0.48^{b}	1.30^{a}	1.37^{a}	1.79^{a}	2.33^{a}	1.69^{a}	1.56^{a}
HP	0.58^{b}	0.25^{b}	0.03^{b}	0.23^{b}	0.60^{c}	0.30^{b}	0.71^{b}	1.05^{b}	0.88^{b}	0.83^{b}
SEM	0.09	0.10	0.06	0.07	0.10	0.13	0.15	0.20	0.17	0.17
P-value	0.0001	0.0001	0.0001	0.0001	0.0001	0.003	0.0001	0.0001	0.0001	0.0001
Treatment										
Vehicle	0.55	0.50	0.35	0.55	1.02	1.31	1.25	1.66	1.25	1.16
NPY	0.72	0.63	0.49	0.62	0.88	1.75	1.05	1.25	1.90	1.16
SEM	0.07	0.08	0.05	0.06	0.83	0.32	0.12	0.16	0.14	0.14
P-value	0.59	0.30	0.17	0.25	0.39	0.26	0.39	0.43	0.48	0.73
D x T	0.90	0.69	0.04	0.70	0.85	0.76	0.60	0.83	0.78	0.45

[1]Main effects of diet (high carbohydrate: HC; high fat: HF; and high protein: HP), treatment (0 (vehicle) or 0.20 nmol NPY (NPY)), and the interaction between diet (D) and treatment (T) on mRNA abundance (n=10 chicks per group). Values represent least squares means and pooled standard errors of the means with associated *P*-values for each effect. Different superscripts within an effect for each gene are significantly different at $P < 0.05$, Tukey's test. Significant two-way interactions are displayed graphically. Abbreviations: CCAAT/enhancer-binding protein alpha and beta: C/EBPα and C/EBPβ, respectively; peroxisome proliferator-activated receptor gamma: PPARγ; sterol regulatory element-binding transcription factor 1: SREBP1; lipoprotein lipase: LPL; adipose triglyceride lipase: ATGL; perilipin 1: PLN1; comparative gene identification-58: CGI58; monoglyceride lipase: MGLL; fatty acid synthase: FASN.

Table 4-4. Effects of diet and treatment on abdominal fat mRNA abundance of adipogenesis-associated factors and proliferation markers.

Effect[1]	ACADL	AGPAT9	FABP4	NPY	NPYR2	Ki67	TOP2A	TPX2	KLF7	GATA2
Diet										
HC	1.08^{b}	1.04^{b}	0.98^{a}	1.06^{a}	1.42^{ab}	0.84^{a}	0.97^{a}	1.01^{a}	0.93^{a}	0.98^{a}
HF	1.77^{a}	2.62^{a}	0.33^{b}	0.48^{b}	1.51^{a}	1.02^{a}	1.10^{a}	1.01^{a}	0.26^{b}	0.44^{b}
HP	0.97^{b}	0.86^{b}	0.38^{b}	0.16^{b}	0.44^{b}	0.38^{b}	0.51^{b}	0.44^{b}	0.41^{b}	0.70^{ab}
SEM	0.19	0.24	0.09	0.09	0.21	0.08	0.08	0.10	0.09	0.14
P-value	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001
Treatment										
Vehicle	1.25	1.66	0.47	0.53	1.01	0.81	0.89	0.85	0.48	0.58
NPY	1.29	1.36	0.64	0.60	1.05	0.69	0.84	0.78	0.58	0.82
SEM	0.15	0.20	0.77	0.08	0.17	0.06	0.07	0.08	0.07	0.11
P-value	0.32	0.82	0.21	0.56	0.52	0.24	0.84	0.68	0.18	0.59
D x T	0.98	0.58	0.03	0.89	0.92	0.08	0.66	0.86	0.17	0.23

[1]Main effects of diet (high carbohydrate: HC; high fat: HF; and high protein: HP), treatment (0 (vehicle) or 0.2 nmol NPY (NPY)), and the interaction between diet (D) and treatment (T) on mRNA abundance (n=10 chicks per group). Values represent least squares means and pooled standard errors of the means with associated *P*-values for each effect. Different superscripts within an effect for each gene are significantly different at $P < 0.05$, Tukey's test. Significant two-way interactions are displayed graphically. Abbreviations: acyl-CoA dehydrogenase, long chain: ACADL; 1-acylglycerol-3-phosphate O-acyltransferase 9: AGPAT9; fatty acid binding protein 4: FABP4; neuropeptide Y: NPY; NPY receptor 2: NPYR2; Ki67; topoisomerase II alpha: TOP2A; targeting protein for Xklp2: TPX2; krüppel-like factor 7: KLF7 and GATA-binding protein 2: GATA2.

Table 4-5. Effects of diet and treatment on subcutaneous fat mRNA abundance of transcription factors and lipid metabolic enzymes.

Effect[1]	C/EBPα	C/EBPβ	PPARγ	SREBP1	LPL	ATGL	MGLL	PLN1	CGI58	FASN
Diet										
HC	1.29[a]	0.79	0.90[a]	0.96[a]	0.89[ab]	1.02[ab]	1.11[b]	0.98[b]	0.96[b]	0.96[b]
HF	0.29[b]	0.78	0.20[b]	0.45[b]	1.22[a]	1.47[a]	2.03[a]	2.02[a]	2.30[a]	1.44[a]
HP	0.54[b]	0.86	0.15[b]	0.22[c]	0.83[b]	0.69[b]	0.81[b]	0.83[b]	0.91[b]	0.75[b]
SEM	0.10	0.11	0.05	0.05	0.08	0.20	0.19	0.25	0.20	0.14
P-value	0.0001	0.89	0.0001	0.0001	0.03	0.0001	0.0001	0.0001	0.0001	0.0003
Treatment										
Vehicle	0.71	0.77	0.41	0.54	0.96	1.12	1.27	1.22	1.37	1.10
NPY	0.74	0.85	0.44	0.56	0.99	0.99	1.36	1.34	1.40	1.17
SEM	0.08	0.09	0.04	0.04	0.10	0.16	0.16	0.15	0.20	0.16
P-value	0.56	0.090	0.87	0.65	0.55	0.92	0.68	0.55	0.97	0.23
D x T	0.13	0.18	0.006	0.002	0.30	0.57	0.94	0.30	0.29	0.06

[1]Main effects of diet (high carbohydrate: HC; high fat: HF; and high protein: HP), treatment (0 (vehicle) or 0.2 nmol NPY (NPY)), and the interaction between diet (D) and treatment (T) on mRNA abundance (n=10 chicks per group). Values represent least squares means and pooled standard errors of the means with associated *P*-values for each effect. Different superscripts within an effect for each gene are significantly different at $P < 0.05$, Tukey's test. Significant two-way interactions are displayed graphically. Abbreviations: CCAAT/enhancer-binding protein alpha and beta: C/EBPα and C/EBPβ, respectively; peroxisome proliferator-activated receptor gamma: PPARγ; sterol regulatory element-binding transcription factor 1: SREBP1; lipoprotein lipase: LPL; adipose triglyceride lipase: ATGL; perilipin 1: PLN1; comparative gene identification-58: CGI58; monoglyceride lipase: MGLL; fatty acid synthase: FASN.

Table 4-6. Effects of diet and treatment on subcutaneous fat mRNA abundance of adipogenesis-associated factors and proliferation markers.

Effect[1]	ACADL	AGPAT9	FABP4	NPY	NPYR2	Ki67	TOP2A	TPX2	KLF7	GATA2
Diet										
HC	0.95^{b}	0.89^{ab}	1.26^{a}	1.02^{a}	1.14^{a}	0.95^{a}	1.16^{a}	1.00^{a}	1.00^{a}	1.06^{a}
HF	1.69^{a}	1.39^{a}	0.38^{b}	0.30^{b}	1.12^{a}	0.57^{b}	0.81^{ab}	0.56^{b}	0.31^{b}	0.40^{b}
HP	0.88^{b}	0.68^{b}	0.50^{b}	0.10^{b}	0.57^{b}	0.33^{b}	0.56^{b}	0.39^{b}	0.93^{a}	1.20^{a}
SEM	0.18	0.18	0.09	0.07	0.16	0.09	0.12	0.08	0.13	0.14
P-value	0.0001	0.0001	0.0001	0.0001	0.02	0.0001	0.0003	0.0001	0.0001	0.0002
Treatment										
Vehicle	1.20	0.94	0.67	0.48	0.90	0.66	0.82	0.66	0.67	0.79
NPY	1.14	1.01	0.78	0.46	0.99	0.57	0.87	0.62	0.84	1.02
SEM	0.15	0.15	0.07	0.05	0.13	0.07	0.09	0.07	0.11	0.11
P-value	0.65	0.88	0.87	0.31	0.60	0.24	0.92	0.67	0.24	0.10
D x T	0.22	0.63	0.37	0.06	0.70	0.17	0.29	0.62	0.37	0.41

[1]Main effects of diet (high carbohydrate: HC; high fat: HF; and high protein: HP), treatment (0 (vehicle) or 0.2 nmol NPY (NPY)), and the interaction between diet (D) and treatment (T) on mRNA abundance (n=10 chicks per group). Values represent least squares means and pooled standard errors of the means with associated *P*-values for each effect. Different superscripts within an effect for each gene are significantly different at $P < 0.05$, Tukey's test. Significant two-way interactions are displayed graphically. Abbreviations: acyl-CoA dehydrogenase, long chain: ACADL; 1-acylglycerol-3-phosphate O-acyltransferase 9: AGPAT9; fatty acid binding protein 4: FABP4; neuropeptide Y: NPY; NPY receptor 2: NPYR2; Ki67; topoisomerase II alpha: TOP2A; targeting protein for Xklp2: TPX2; krüppel-like factor 7: KLF7 and GATA-binding protein 2: GATA2.

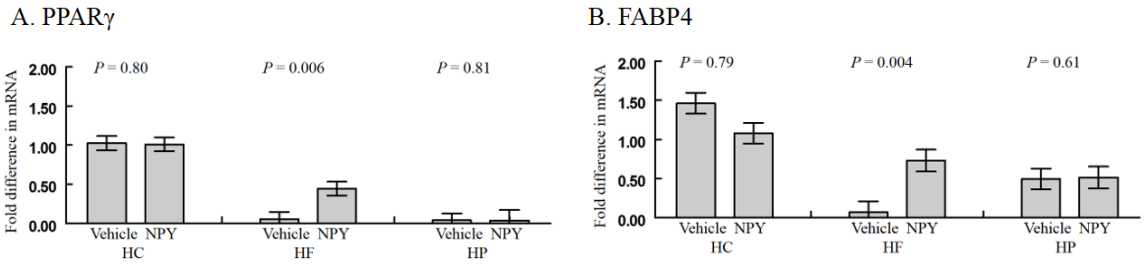

Figure 4-1. Interactions of diet and neuropeptide Y treatment on mRNA abundance in abdominal fat. A) peroxisome proliferator-activated receptor gamma (PPARγ; $P = 0.04$) and B) fatty acid binding protein 4 (FABP4; $P = 0.03$). At day 4 post-hatch, chicks fed one of three diets (HC: high carbohydrate; HF: high fat; HP: high protein) were centrally-injected with vehicle or 0.2 nmol NPY (n=10 chicks per group). Interactions were separated using the Slice function of JMP Pro 12 (SAS Inst.) and significant slices further analyzed by secondary ANOVA with the model including the main effect of treatment within each dietary group. Different letters within a dietary group indicate a significant difference at $P < 0.05$; Tukey's test.

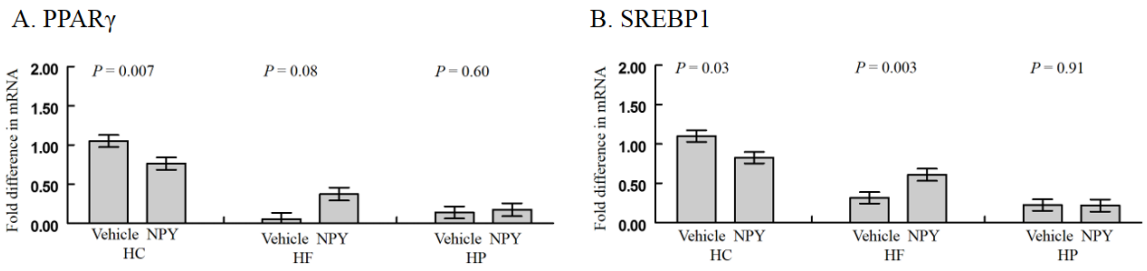

Figure 4-2. Interactions of diet and neuropeptide Y treatment on mRNA abundance in subcutaneous fat. A) peroxisome proliferator-activated receptor gamma (PPARγ; $P = 0.006$) and B) sterol regulatory element-binding transcription factor 1 (SREBP1; $P = 0.002$). At day 4 post-hatch, chicks fed one of three diets (HC: high carbohydrate; HF: high fat; HP: high protein) were centrally-injected with vehicle or 0.2 nmol NPY (n=10 chicks per group). Interactions were separated using the Slice function of JMP Pro 12 (SAS Inst.) and significant slices further analyzed by secondary ANOVA with the model including the main effect of treatment within each dietary group. Different letters within a dietary group indicate a significant difference at $P < 0.05$; Tukey's test.

Chapter 5: Responses to peripheral neuropeptide Y in avian adipose tissue are diet, depot, and time specific

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Abstract

The goal of this research was to determine the effect of dietary macronutrient composition on peripheral neuropeptide Y (NPY)-induced changes in adipose tissue dynamics in chicks. Chicks were fed one of three isocaloric diets from the day of hatch: high carbohydrate (HC), high fat (HF), or high protein (HP). On day 4 post-hatch, 0 (vehicle), 60, or 120 µg/kg BW of NPY was injected intraperitoneally, and subcutaneous, clavicular and abdominal adipose tissue samples were collected at 1 and 3 h post-injection. The effect of NPY was most pronounced in chicks fed the HF or HP diet. In the subcutaneous fat at 1 h post-injection, 60 µg/kg BW of NPY was associated with an increase in NPY receptor 2 (NPYR2) mRNA in chicks fed the HP diet and a decrease in 1-acylglycerol-3-phosphate O-acyltransferase 2 (AGPAT2) mRNA in chicks fed the HC diet. In response to 120 µg/kg BW of NPY, there was greater AGPAT2 mRNA in the clavicular fat of chicks that consumed the HP diet and less CCAAT/enhancer-binding protein alpha in the abdominal fat of chicks that were provided the HF diet. There were no gene expression changes in the abdominal fat at 3 h post-injection, whereas there were decreases in AGPAT2, adipose triglyceride lipase, fatty acid binding protein 4 and NPY mRNA in the clavicular fat of chicks fed the HP diet. Results demonstrate that diet affects exogenous NPY-dependent physiological effects in a time- and depot-dependent manner in chick adipose tissue.

Keywords: adipogenesis; adipose; chick; dietary macronutrient; neuropeptide Y; mRNA

Introduction

Neuropeptide Y (NPY) is one of the most potent stimulators of food intake across a variety of species, including mammals (206) and birds (89). The orexigenic effect of NPY is influenced by other factors, such as dietary macronutrient composition. There is a reciprocal relationship as diet composition affects NPY release and NPY in turn influences the selection of specific diets that vary in nutrient composition. For example, when provided free-choice access to high carbohydrate (HC), high fat (HF) or high protein (HP) diets, rats have a preference for the HC diet over the other two diets following central NPY injection (226). Relative to rats fed a control diet, consumption of a HC diet increased mRNA abundance of hypothalamic NPY (227).

Neuropeptide Y is one of the most abundant neurotransmitters in the central nervous system and is also detectable in peripheral regions, including the plasma, sympathetic nerves that innervate the adipose tissue (AT), and various cell types within the white AT (228). Similar to the NPY concentrations in the hypothalamus, NPY abundance in the AT may be affected by dietary macronutrient composition. For instance, consumption of a HF diet is associated with increased NPY transcript quantities in the visceral fat of rats (229).

In mammals, NPY has hyperplasic, adipogenic and anti-lipolytic effects in adipose tissue (91, 230). The mechanisms mediating these effects in mammals are partly understood, however, little is known in avian species. We showed that gene expression of NPY and its three receptor sub-types 1 (NPYR1), NPYR2 and NPYR5 is greater in abdominal fat from chickens that become obese late in life as compared to those that are anorexic and relatively lean (231). In addition, we reported that NPY promotes adipogenesis in chicken adipocyte precursor cells *in vitro* (95) and *in vivo* from the obese and anorexic chickens (232). Therefore, the purpose of the present study

was to expand knowledge in this area by determining how dietary macronutrient composition and intraperitoneal (IP) injection of NPY affect AT physiology in chicks.

Materials and methods

Experimental animals

All animal protocols were approved by the Institutional Animal Care and Use Committee at Virginia Tech and animals were cared for in accordance with the Guide for the Care and Use of Laboratory Animals. Day of hatch Cobb-500 broiler chicks were obtained from a local hatchery and housed in electrically heated and thermostatically controlled cages with ad libitum access to food and water. The temperature was 30 ± 2 ℃ with 50 ± 5% relative humidity and 24 h of light. Diets were formulated to be isocaloric (3,000 kcal/kg) as shown in Table 5-1 and mixed at Augusta Cooperative Feed Mill (Staunton, Virginia, USA). The high carbohydrate (HC) diet was formulated to meet the minimum requirements defined for the starter phase of commercial broilers (http://www.cobb-vantress.com) and serves as a broiler industry standard starter diet. The high protein (HP) diet was 25% crude protein and the high fat (HF) diet was formulated to have 30% of the metabolizable energy derived from calories in soybean oil. Crude protein and fat values were experimentally verified for all diets (Table 5-1).

Experiment 1: Food intake

Chicks were randomly assigned to one of the three diets at day of hatch, with ad libitum access to food and water. At day 4 post-hatch, chicks from each diet were randomly assigned to IP injection of one of three treatments: 0 (phosphate-buffered saline), 60 or 120 µg/kg BW NPY with n = 20 chicks per group. Doses were determined through preliminary experiments, with the lower dose being one at which an effect on glycerol-3-phosphate dehydrogenase activity and

mRNA abundance in adipose tissue was observed at 1 hour post-injection (232), also within the range used in similar studies with rat and mouse models (229, 233). Day 4 was selected because it is the age at which our other studies have been conducted that examined effects of peripheral (232) and central (234) NPY on adipose tissue function and feeding behavior (207), and represents the beginning of abdominal fat deposition in broiler chicks (174). Additionally, we have evaluated the effects of dietary macronutrient composition (same diets used in present study) and fasting and refeeding on adipose tissue function (202) at day 4 post-hatch. Chicks were euthanized at 1 and 3 h post-injection for tissue collection (n = 10 at each time point) and sexed by visual inspection of gonads. Food intake was measured (to 0.01 g) at 1 and 3 h post-injection.

Experiment 2: Total RNA isolation and real-time PCR

Clavicular, subcutaneous and abdominal ATs were collected as previously described (174) and submerged in RNAlater (Qiagen). Tissues were homogenized in 1 mL Tri-Reagent (Molecular Research Center, TR 118) using 5 mm stainless steel beads (Qiagen, Valencia, CA, USA) and a Tissue Lyser II (Qiagen) for 2 × 2 min at 25 Hz. Total RNA was separated following the manufacturer's instructions and following the step of addition to molecular biology-grade ethanol, samples were transferred to spin columns and further purified using Direct-zol RNA Kits (Zymo Research) with the optional RNase-free DNase I (Zymo Research) treatment. Single-strand cDNA was synthesized with a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) as described (174). Primers for real time PCR were designed with Primer Express 3.0 software (Applied Biosystems), and validated for having similar (within 5% of reference gene) amplification efficiency before use (Table 5-2). Real-time PCR was performed as we described (174).

Experiment 3: Plasma non-esterified fatty acid concentrations

Approximately 200 µL blood was collected from the trunk (n = 10 chicks) via capillary blood collection tubes (Microvette®) immediately following euthanasia and decapitation. After collection, blood samples were centrifuged at 2,000 g at room temperature and plasma was isolated. Plasma non-esterified fatty acid (NEFA) concentrations were measured using the NEFA-HR2 kit (Wako Diagnostics) according to the manufacturer's instructions. Absorbance was measured at 550 nm using an Infinite M200Pro multi-mode plate reader (Tecan). Sample concentration was calculated using the following formula: Sample Concentration = Standard Concentration × (Sample Absorbance) /(Standard Absorbance). Concentrations are reported as mEq/L.

Statistical analysis

The real time PCR data were analyzed using the ΔΔCT method, where ΔCT = CT target gene − CT actin, and ΔΔCT = ΔCT target sample − ΔCT calibrator (175). The average of subcutaneous fat from chicks fed the HC diet that received the vehicle treatment was used as the calibrator sample. The fold difference (relative quantity; RQ) was calculated as $2^{-\Delta\Delta CT}$. Analysis of variance (ANOVA) was performed for food intake, NEFA concentrations, and RQ values within each diet at each time point using the Fit Model platform of JMP Pro11 (SAS Institute, Cary, NC). For food intake and RQ values, ANOVAs were performed within adipose tissue depot. Because sex and interactions between sex and NPY treatment did not significantly influence any of the traits measured in this study, sex was excluded from the statistical models. The model included the main effect of NPY treatment. Tukey's test was used to separate the means. Differences were considered significant at $P < 0.05$.

Data were transformed (212) as follows to reduce heterogeneity of variance, where x is the RQ value: 1/x: 1-acylglycerol-3-phosphate O-acyltransferase 2 (AGPAT2) (subcutaneous 1h,

clavicular 1h, 3h, abdominal 1h), CCAAT/enhancer-binding protein beta (C/EBPβ) (subcutaneous 1h, 3h, abdominal 1h), fatty acid binding protein 4 (FABP4) (subcutaneous 1h 3h), NPY (subcutaneous 1h, clavicular 1h, abdominal 1h), diglyceride acyltransferase 2 (DGAT2) (subcutaneous 3h, clavicular 3h, abdominal 1h, 3h), C/EBPα (subcutaneous 3h, abdominal 1h, 3h), lipoprotein lipase (LPL) (subcutaneous 3h, abdominal 3h), peroxisome proliferator-activated receptor gamma (PPARγ) (subcutaneous 3h, clavicular 3h, abdominal 1h), sterol regulatory element-binding transcription factor 1 (SREBP1) (subcutaneous 3h, abdominal 3h) adipose triglyceride lipase (ATGL) (clavicular 3h) ; lnx: ATGL (subcutaneous 1h 3h), NPY (abdominal 3h), NPYR2 (abdominal 3h), PPARγ (abdominal 3h), SREBP1 (abdominal 3h); 1/(1+x): C/EBPα (subcutaneous 1h), NPYR2 (subcutaneous 1h), LPL (subcutaneous 1h); log10(x): PPARγ (subcutaneous 1h), SREBP1 (subcutaneous 1h), ATGL (clavicular 1h), SREBP (clavicular 3h, abdominal 1h), LPL (abdominal 1h); sqrt(x)+sqrt(x+1): DGAT2 (clavicular 1h), SREBP1 (clavicular 1h), FABP4 (abdominal 3h); log10(x+1): NPYR2 (clavicular 1h), C/EBPα (clavicular 3h), FABP4 (clavicular 3h, abdominal 1h), NPYR2 (clavicular 3h), ATGL (abdominal 1h, 3h); sqrt(x+0.5): NPYR2 (abdominal 1h); ln(x+1): AGPAT2 (abdominal 3h).

Results

Food intake

Peripheral NPY treatment did not affect food intake in chicks fed any of the diets at 1 or 3 h post-injection (Figure 5-1).

mRNA abundance in adipose tissue

The mRNA abundance results for subcutaneous, clavicular and abdominal AT are summarized in Tables 5-3, 5-4, and 5-5, respectively.

Subcutaneous fat

At 1 h post-injection, there was decreased expression of AGPAT2 in chicks fed the HC diet that received 60 µg/kg NPY compared to chicks from the same dietary group that received the vehicle (Table 5-3a). Expression of NPYR2 was greater in chicks that consumed the HP diet and received the lower dose of NPY as compared to chicks from the same dietary group that received the vehicle or higher dose.

At 3 h post-injection, C/EBPβ mRNA was greater in chicks fed the HF diet that received 60 than chicks that received 120 µg/kg NPY (Table 5-3b). Both 60 and 120 µg/kg NPY injection increased the expression of SREBP1 in chicks fed the HP diet.

Clavicular fat

At 1 h post-injection, the expression of AGPAT2 was greater in chicks fed the HP diet that received the higher dose of NPY than in those that were injected with the vehicle (Table 5-4a). At 3 h, there was greater NPY mRNA in HF diet-fed chicks that received the lower dose of NPY than vehicle (Table 5-4b). The higher dose of NPY decreased the expression of FABP4 at 3 h post-injection in chicks fed the HP diet compared to chicks that received the vehicle. At 3 h, there was greater expression of AGPAT2, ATGL, and NPY in vehicle- than NPY-injected chicks.

Abdominal fat

At 1 h, the expression of C/EBPα was lower in chicks fed the HF diet that received 120 µg/kg NPY than in chicks that received the vehicle or lower dose (Table 5-5a). In chicks that consumed the HF diet, FABP4 and PPARγ mRNAs were greater in the low dose- than high dose-injected group. There were no effects of NPY on mRNA abundance in the abdominal fat at 3 h post-injection (Table 5-5b).

Plasma NEFA concentrations

There was no effect of NPY treatment on plasma NEFAs in any of the dietary groups at either 1 or 3 h post-injection (Figure 5-2).

Discussion

According to our previous studies using the same diets, dietary treatments did not affect body weight and food intake of the chicks, whereas the HP diet decreased adipose tissue weight (202). In addition, the expression of adipogenesis-associated factors was reduced in the AT of 4 day-old chicks fed the HF or HP diet relative to chicks that consumed the HC diet (202). Those studies involved the effects of fasting and refeeding (202) and effects of central injection of NPY (234) and chicks were sampled at the same age as in the present study. When two-way ANOVA was used to analyze the data in the present study, we found that the effect of diet on body weight gain, food intake and the expression of adipogenesis-associated factors was similar to our previous studies (202). Thus, data from the present study were analyzed within each diet to focus on the effect of IP NPY injection on adipose tissue physiology.

Based on mammalian studies, NPY stimulates preadipocyte proliferation and differentiation (91). Our group also reported that NPY promotes adipogenesis during the early and later stages of chicken preadipocyte differentiation (95, 96). In addition, NPY exerts its functions by binding to a variety of NPY receptors (NPYRs), with the most well-known ones, NPYR1, NPYR2 and NPYR5, being associated with both appetite- and adipose physiology-related effects (235). The effects of NPY on AT adipogenesis are mediated mainly through NPYR2 in mice (91, 92). Also, in our studies with chicks, NPYR2 was the receptor sub-type for which we were able to detect mRNA in both whole AT and isolated stromal-vascular fraction cells (95, 96, 231). Thus, only

the expression of NPYR2 was evaluated in the present study. The abundance of NPYR2 mRNA was increased at 1 h after NPY injection (lower dose) in the subcutaneous fat of chicks fed the HP diet. There was an increase and decrease in NPY expression at 3 h in the clavicular fat of chicks fed the HF and HP diets, respectively. In mice, the release of NPY from sympathetic nerves in turn upregulated NPY and NPYR2, resulting in the expansion of abdominal fat (91). Thus, in the present study the injected NPY might have provided positive feedback for the increase in NPY and NPYR2 mRNA in the AT of chicks. In the HP diet group in clavicular fat at 3 h post-injection, all significant changes were reductions in mRNA abundance in response to NPY, whereas in abdominal fat no changes were detected at 3 h and in subcutaneous fat there were a few in the form of increases in the HF and HP dietary groups.

Peripheral NPY injection was associated with an increase in the expression of AGPAT2 in the clavicular fat of chicks fed the HP diet at 1 h and the expression of SREBP1 in the subcutaneous fat of chicks fed the HF diet at 3 h post-injection. The AGPAT2 is an enzyme that catalyzes the synthesis of phosphatidic acid, a key intermediate in the biosynthesis of triacylglycerol and glycerophospholipids, via the acylation of lysophosphatidic acid (236). The SREBP1 is a key transcription factor during the early stages of adipogenesis (183) that promotes adipocyte differentiation in mammals (215) and chickens (237). Thus, results suggest that NPY enhances adipogenesis and triacylglycerol synthesis via effects on transcriptional regulation of lipid metabolic enzymes and transcription factors.

However, NPY injection also decreased the expression of AGPAT2, ATGL, NPY and FABP4 in the clavicular fat of chicks fed the HP diet at 3 h, and C/EBPα, FABP4 and PPARγ in the abdominal fat of chicks fed the HF diet at 1 h post-injection. Our *in vitro* studies also showed that NPY treatment decreased the expression of C/EBPα and SREBP1 at day 4 post-induction of

differentiation and PPARγ at day 4 and 6 post-induction of differentiation, however, the expression of these genes was recovered at day 8 post-induction of differentiation (95, 96). One may argue that the amount of NPY treatment time during the *in vitro* studies was substantially longer than the current study and that 3 hours was not long enough for there to be an effect of NPY treatment on transcriptional regulation. However, we recently found that a single IP NPY injection (same doses and similar age) in a different chicken model influenced mRNA abundance of adipogenesis-associated factors at 1 and 3 h post-injection and that effects were diminished at 12 h post-injection (232). Because of the differences in experimental models between the *in vivo* and *in vitro* studies, it is unclear how to compare the effective time of treatment. For example, in the *in vitro* study, NPY has direct access to adipocytes, however, NPY was injected into the peritoneum in the present study and it is unclear how much would have reached the adipocytes, and how other cell types in the AT would affect the effect of NPY.

In our previous study, abdominal fat weight and abdominal adipocyte area/diameter were less than in subcutaneous and clavicular fat at 4 days of age (202). In the present study, not one of the genes measured was increased in the abdominal fat of chicks after IP NPY injection, which may further indicate that abdominal fat is less responsive on a transcriptional level to stimuli that induce adipogenesis and lipid metabolism at an early age, although it is the depot that develops the most on a percent of body weight-basis between day 4 and 14 post-hatch (174).

We also observed in a previous study that there was greater expression of PPARγ, C/EBPα, and SREBP1 in the subcutaneous and abdominal fat of chicks fed the HC diet than in chicks fed the HF and HP diets (202). In the present study, IP NPY injection did not increase the expression of any adipogenesis- and lipid metabolism-associated genes in the three AT depots of chicks fed the HC diet, whereas an increase in some of these genes occurred in chicks fed the HF or HP diet.

Because the basal expression levels of some of these genes are higher in the AT of chicks fed the HC diet, they might be less responsive to the effect of NPY.

In murine white AT, ATGL and hormone sensitive lipase (HSL) are the major enzymes involved in triacylglycerol (TG) catabolism, accounting for more than 95% of the hydrolase activity (195). However, no orthologue of HSL has been identified in the chicken genome (45), suggesting that ATGL plays an even more important role in lipolysis in chickens. Adipose triglyceride lipase hydrolyzes the first ester bond of a TG to release a fatty acid and diacylglycerol, acting as the rate-limiting step in lipolysis (39, 40, 238). Depot-specific regulation of lipolysis also exists, for example, visceral and subcutaneous abdominal ATs are more responsive to lipolytic stimuli, such as catecholamines or prolonged fasting, than subcutaneous gluteal and femoral fat (239, 240). In the present study, there was reduced expression of ATGL in the clavicular fat of chicks that received NPY and consumed the HP diet. Because clavicular fat was the only AT depot that responded to IP NPY injection, the anti-lipolytic effect of NPY on the clavicular fat might be diluted at the whole animal level. This might explain why IP NPY injection did not decrease plasma NEFAs in chicks fed the different diets.

In the periphery, NPY colocalizes with norepinephrine and is released from sympathetic nerve terminals upon sympathetic stimulation, and the sympathetic nervous system innervates the white AT (241, 242). In addition to the NPY released from sympathetic nerve endings, cells within the white adipose tissue and circulation are also sources of NPY, however, the relative contribution to the total NPY concentration in the WAT is still unclear (91, 243). Although circulating NPY is able to cross the blood-brain barrier (244) and the concentration of injected NPY is much higher than amounts in the cerebrospinal fluid (CSF) (245), food intake was not increased at 1 or 3 h post-injection, irrespective of the diet. This suggests that effects on AT

physiology were independent of secondary effects due to altered food intake. In humans there was a weak correlation between NPY concentrations in the CSF and plasma (246). In addition, the half-life for NPY is also short as compared with steroid neurotransmitters, with a half-life of up to 39 min in adult men (247). Thus, local blood supply and sympathetic nervous system innervation may influence the response of AT depots to the adipogenic and/or anti-lipolytic effects of NPY.

Conclusions

In summary, peripheral NPY injection did not affect food intake or plasma NEFA concentrations in chicks fed the HC, HF or HP diet. There was an increase in mRNA abundance of some adipogenesis-associated genes in the subcutaneous and clavicular fat of chicks fed the HF and HP diets, suggesting a greater responsiveness of these depots and dietary groups to the adipogenic effects of NPY. However, IP NPY injection also decreased the expression of some of these factors, although mechanisms are still unclear. These results may provide implications for understanding how NPY affects development of different adipose depots during the early-life period and mechanisms underlying depot-dependent and diet-dependent differences in metabolism across species.

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Table 5-1. Ingredient and chemical composition of experimental diets.

Ingredient (% as fed)[a]	High carbohydrate	High protein	High fat
Corn grain	60.37	43.61	31.28
Soybean meal	33.99	48.8	36.94
Soy hulls	0	0	17.27
Dicalcium phosphate	1.5	1.42	1.56
Limestone	1.21	1.14	0.89
Soybean oil	1.13	3.65	10.20
Vitamin/mineral premix[b]	1	1	1
Methionine99%	0.26	0.12	0.32
L-Lysine HCL78%	0.19	0	0.16
Sodium bicarbonate	0.15	0.15	0.15
L-Threonine	0.09	0	0.12
Coban 90[c]	0.05	0.05	0.05
Choline-Cl60%	0.05	0.05	0.05
Phytase-Ronozyme-10000[d]	0.01	0.01	0.01
Kcal ME/kg	3,000	3,000	3,000
Crude protein[e]	22%	25%	22%
Crude fat[e]	3.0%	4.6%	10.2%

[a]Diets were formulated to meet or exceed minimum recommended specifications for Cobb-500 broilers during the starter phase (Cobb-Vantress).

[b]Guaranteed analysis (per kg of premix): Manganese, 25.6 g; selenium, 120 mg; zinc, 30 g; Vitamin A, 4409, 171.076 IU; Vitamin D3, 1410,934.744 ICU; 13,227.513 IU; d-biotin, 88.183 mg.

[c]Coban 90 (Elanco Animal Health) contains 90 g of Monensin sodium per pound of premix and is included in the diet as a coccidiostat.

[d]DSM Nutritional Products, Ltd.

[e]Analyzed at Experiment Station Chemical Laboratories at University of Missouri

Table 5-2. Primers used for real time PCR.

Gene[1]	Primers sequence (5'-3'); Forward/Reverse	Accession No.
β-actin	GTCCACCGCAAATGCTTCTAA/TGCGCATTTATGGGTTTTGTT	NM_205518.1
AGPAT2	GCCAAACACCGAAGGAACAT/CCATGGCATCCCCAGAGTT	XM_015279793.1
ATGL	GCCTCTGCGTAGGCCATGT/GCAGCCGGCGAAGGA	NM_001113291.1
DGAT2	TTGGCTTTGCTCCATGCAT/CCCACGTGTTCGAGGAGAA	XM_419374.5
C/EBPα	CGCGGCAAATCCAAAAAG/GGCGCACGCGGTACTC	NM_001031459.1
C/EBPβ	GCCGCCCGCCTTTAAA/CCAAACAGTCCGCCTCGTAA	NM_205253.2
FABP4	CAGAAGTGGGATGGCAAAGAG/CCAGCAGGTTCCCATCCA	NM_204290.1
NPY	CATGCAGGGCACCATGAG/CAGCGACAAGGCGAAAGTC	M87294.1
NPYR2	TGCCTACACCCGCATATGG/TTGTCTTCTGCTTGCGTTTTGT	NM_001031128.1
LPL	GACAGCTTGGCACAGTGCAA/CACCCATGGATCACCACAAA	NM_205282.1
PPARγ	CACTGCAGGAACAGAACAAAGAA/TCCACAGAGCGAAACTGACATC	NM_001001460.1
SREBP1	CATCCATCAACGACAAGATCGT/CTCAGGATCGCCGACTTGTT	NM_204126.1

[1]Gene abbreviations: 1-acylglycerol-3-phosphate O-acyltransferase 2: AGPAT2; adipose triglyceride lipase: ATGL; diacylglycerol O-acyltransferase 2: DGAT2; CCAAT/enhancer-binding protein alpha and beta: C/EBPα and C/EBPβ, respectively; fatty acid binding protein 4: FABP4; neuropeptide Y: NPY; NPY receptor 2: NPYR2; lipoprotein lipase: LPL; peroxisome proliferator-activated receptor gamma: PPARγ; sterol regulatory element-binding transcription factor 1: SREBP1.

Table 5-3a. Subcutaneous fat mRNA abundance at 1 h post-injection.

Effect[1]	AGPAT2	ATGL	DGAT2	C/EBPα	C/EBP$_\beta$	FABP4	NPY	NPYR2	LPL	PPARγ	SREBP1
HC Diet											
Vehicle	1.02[a]	1.12	1.00	1.02	1.02	1.08	0.88	0.90	1.03	1.06	1.08
60	0.75[b]	0.90	0.87	0.80	0.91	0.77	0.44	0.37	0.93	0.94	0.82
120	0.84[ab]	1.00	0.93	0.78	1.02	0.81	0.47	0.44	0.96	0.82	0.94
SEM	0.064	0.19	0.19	0.073	0.092	0.11	0.12	0.19	0.087	0.12	0.14
P-value	0.02	0.72	0.90	0.06	0.63	0.13	0.04	0.14	0.69	0.42	0.43
HF Diet											
Vehicle	0.68	0.39	1.09	0.68	0.78	0.34	0.60	0.22	0.97	1.19	0.83
60	0.79	0.72	0.96	0.64	0.99	0.36	0.69	0.30	0.87	0.83	0.86
120	0.65	0.40	0.91	0.65	0.86	0.34	0.53	0.26	1.07	1.12	0.85
SEM	0.062	0.13	0.18	0.079	0.14	0.041	0.14	0.051	0.095	0.14	0.12
P-value	0.30	0.16	0.77	0.94	0.59	0.93	0.72	0.54	0.36	0.18	0.98
HP Diet											
Vehicle	0.71	0.55	1.36	0.83	0.85	0.25	0.67	0.25[b]	1.34	0.93	1.06
60	0.93	1.02	0.84	0.80	1.10	0.26	1.17	1.10[a]	1.27	0.96	1.02
120	0.96	0.82	1.04	0.82	1.087	0.24	0.76	0.37[b]	1.29	0.80	1.34
SEM	0.072	0.16	0.23	0.079	0.098	0.019	0.18	0.18	0.12	0.10	0.15
P-value	0.04	0.13	0.32	0.96	0.15	0.86	0.15	0.008	0.90	0.55	0.27

[1]Values represent least squares means and pooled standard errors of the means with associated *P*-values for the effect of NPY treatment within diet and gene. Different superscripts within an effect for each gene are significantly different at $P < 0.05$, Tukey's test. At 4 days post-hatch, chicks that were fed the high carbohydrate (HC), high fat (HF), or high protein (HP) diet were injected with vehicle, 60, or 120 µg/kg neuropeptide Y (NPY) and tissue collected at 1 h post-injection. Abbreviations: 1-acylglycerol-3-phosphate O-acyltransferase 2: AGPAT2; adipose triglyceride lipase: ATGL; diacylglycerol O-acyltransferase 2: DGAT2; CCAAT/enhancer-binding protein alpha and beta: C/EBPα and C/EBPβ,

respectively; fatty acid binding protein 4: FABP4; neuropeptide Y: NPY; NPY receptor 2: NPYR2; lipoprotein lipase: LPL; peroxisome proliferator-activated receptor gamma: PPARγ; sterol regulatory element-binding transcription factor 1: SREBP1.

Table 5-3b. Subcutaneous fat mRNA abundance at 3 h post-injection.

Effect[1]	AGPAT2	ATGL	DGAT2	C/EBPα	C/EBP$_\beta$	FABP4	NPY	NPYR2	LPL	PPARγ	SREBP1
HC Diet											
Vehicle	1.18	1.23	1.03	1.02	1.57	0.32	0.62	0.63	1.11	1.19	0.86
60	1.17	1.11	0.78	1.08	1.52	0.28	0.39	0.43	0.94	1.19	0.87
120	1.35	1.16	0.84	1.14	1.31	0.33	0.48	0.37	1.08	1.09	0.92
SEM	0.10	0.26	0.27	0.11	0.17	0.028	0.11	0.17	0.098	0.14	0.14
P-value	0.41	0.94	0.79	0.76	0.52	0.36	0.39	0.55	0.44	0.86	0.96
HF Diet											
Vehicle	0.96	1.24	1.07	0.75	1.38[ab]	0.34	0.44	0.33	1.27	0.95	0.99
60	0.94	1.25	0.50	0.75	1.83[a]	0.28	0.54	0.37	1.16	0.78	0.84
120	0.86	0.90	1.35	0.82	1.10[b]	0.29	0.61	0.38	1.35	1.02	1.06
SEM	0.088	0.25	0.35	0.13	0.15	0.027	0.13	0.069	0.15	0.14	0.18
P-value	0.72	0.53	0.24	0.92	0.01	0.27	0.67	0.84	0.71	0.47	0.70
HP Diet											
Vehicle	0.93	0.76	0.44	0.61	1.39	0.29	0.43	0.33	0.84	0.71	0.64[b]
60	0.77	0.62	1.04	0.78	1.38	0.30	0.54	0.29	0.94	1.02	0.96[a]
120	0.70	0.44	0.98	0.75	1.34	0.27	0.61	0.75	0.73	0.87	0.67[a]
SEM	0.11	0.12	0.26	0.078	0.18	0.029	0.10	0.15	0.092	0.098	0.081
P-value	0.31	0.21	0.22	0.27	0.98	0.73	0.49	0.077	0.31	0.11	0.02

Table 5-4a. Clavicular fat mRNA abundance at 1 h post-injection.

Effect	AGPAT2	ATGL	DGAT2	C/EBPα	C/EBP$_\beta$	FABP4	NPY	NPYR2	LPL	PPARγ	SREBP1
HC Diet											
Vehicle	1.43	0.59	2.80	0.89	0.74	0.23	0.85	0.59	0.82	0.89	1.43
60	1.68	0.80	2.34	1.00	0.89	0.30	1.13	0.59	1.05	0.99	1.70
120	2.03	0.74	2.75	1.03	0.89	0.33	1.06	0.77	1.17	1.00	1.93
SEM	0.22	0.15	0.56	0.15	0.11	0.041	0.20	0.21	0.14	0.16	0.34
P-value	0.18	0.54	0.82	0.79	0.56	0.23	0.61	0.79	0.22	0.86	0.61
HF Diet											
Vehicle	1.17	0.49	1.85	1.12	0.86	0.26	1.43	0.38	1.36	1.26	2.52
60	1.05	0.71	1.39	1.14	0.88	0.29	1.61	0.41	1.06	1.20	1.82
120	0.91	0.48	1.21	1.00	0.80	0.24	1.72	0.50	1.08	1.11	1.93
SEM	0.14	0.11	0.23	0.11	0.090	0.019	0.21	0.18	0.13	0.15	0.25
P-value	0.41	0.23	0.15	0.63	0.82	0.23	0.64	0.88	0.21	0.78	0.15
HP Diet											
Vehicle	0.88[b]	0.52	1.66	1.06	0.84	0.23	1.19	0.44	1.21	1.05	2.08
60	1.03[ab]	0.78	1.02	1.15	0.79	0.28	0.97	0.24	1.18	1.00	1.63
120	1.29[a]	0.69	1.47	1.26	0.89	0.29	1.12	0.46	1.28	1.18	2.17
SEM	0.073	0.14	0.31	0.095	0.11	0.026	0.18	0.20	0.11	0.11	0.21
P-value	0.002	0.47	0.37	0.35	0.83	0.21	0.71	0.72	0.81	0.53	0.21

[1]Values represent least squares means and pooled standard errors of the means with associated *P*-values for the effect of NPY treatment within diet and gene. Different superscipts within an effect for each gene are significantly different at $P < 0.05$, Tukey's test. Abbreviations: 1-acylglycerol-3-phosphate O-acyltransferase 2: AGPAT2; adipose triglyceride lipase: ATGL; diacylglycerol O-acyltransferase 2: DGAT2; CCAAT/enhancer-binding protein alpha and beta: C/EBPα and C/EBPβ, respectively; fatty acid

binding protein 4: FABP4; neuropeptide Y: NPY; NPY receptor 2: NPYR2; lipoprotein lipase: LPL; peroxisome proliferator-activated receptor gamma: PPARγ; sterol regulatory element-binding transcription factor 1: SREBP1.

Table 5-4b. Clavicular fat mRNA abundance at 3 h post-injection.

Effect[1]	AGPAT2	ATGL	DGAT2	C/EBPα	C/EBP$_\beta$	FABP4	NPY	NPYR2	LPL	PPARγ	SREBP1
HC Diet											
Vehicle	1.08	1.25	0.74	4.10	1.51	0.36	0.71	0.20	1.11	1.25	1.55
60	1.11	1.07	0.91	4.00	1.41	0.35	0.82	0.27	1.13	1.37	1.93
120	1.20	1.12	1.10	6.65	1.40	0.41	0.69	0.23	1.33	1.25	2.01
SEM	0.099	0.26	0.25	0.82	0.17	0.047	0.11	0.049	0.13	0.20	0.26
P-value	0.68	0.88	0.61	0.06	0.89	0.65	0.68	0.59	0.44	0.89	0.47
HF Diet											
Vehicle	0.90	0.81	0.82	1.79	1.17	0.29	0.67^{b}	0.19	0.97	1.09	1.28
60	1.01	0.76	0.87	1.45	1.31	0.32	1.22^{a}	0.24	0.98	1.26	1.44
120	1.11	0.54	0.91	0.96	1.40	0.34	0.98^{ab}	0.19	1.00	1.53	1.84
SEM	0.13	0.17	0.27	0.45	0.18	0.038	0.14	0.046	0.16	0.22	0.22
P-value	0.55	0.52	0.97	0.45	0.71	0.71	0.03	0.70	0.99	0.41	0.24
HP Diet											
Vehicle	1.32^{a}	1.40^{a}	0.85	0.99	1.50	0.38^{a}	0.99^{a}	0.24	1.06	1.27	1.54
60	0.77^{b}	0.77^{b}	0.89	0.98	1.32	0.27^{ab}	0.56^{b}	0.13	0.95	1.30	1.53
120	0.66^{b}	0.59^{b}	1.04	0.94	0.97	0.23^{b}	0.57^{b}	0.16	0.86	1.17	1.45
SEM	0.11	0.17	0.25	0.095	0.15	0.032	0.11	0.040	0.082	0.16	0.23
P-value	0.001	0.01	0.86	0.91	0.095	0.01	0.02	0.16	0.29	0.82	0.95

Table 5-5a. Abdominal fat mRNA abundance at 1 h post-injection.

Effect[1]	AGPAT2	ATGL	DGAT2	C/EBPα	C/EBP$_\beta$	FABP4	NPY	NPYR2	LPL	PPARγ	SREBP1
HC Diet											
Vehicle	0.83	1.32	0.68	1.47	1.84	0.22	1.29	1.12	0.52	1.05	1.58
60	0.90	1.46	0.76	1.42	1.91	0.20	1.03	0.95	0.52	0.96	1.51
120	0.78	0.95	0.60	1.10	2.39	0.15	1.26	0.94	0.42	0.81	1.43
SEM	0.12	0.28	0.15	0.19	0.41	0.024	0.33	0.43	0.070	0.090	0.24
P-value	0.81	0.43	0.78	0.37	0.60	0.22	0.83	0.95	0.52	0.18	0.91
HF Diet											
Vehicle	0.85	0.51	0.92	1.07^{a}	2.72	0.19^{ab}	0.93	1.77	0.66	0.91^{ab}	1.85
60	1.00	0.59	0.95	1.18^{a}	2.69	0.22^{a}	1.54	2.30	0.64	1.04^{a}	1.74
120	0.75	0.29	0.51	0.66^{b}	3.46	0.15^{b}	1.17	2.13	0.45	0.63^{b}	1.53
SEM	0.094	0.12	0.17	0.084	0.61	0.014	0.25	0.64	0.069	0.091	0.24
P-value	0.19	0.24	0.14	0.001	0.62	0.007	0.25	0.84	0.084	0.02	0.65
HP Diet											
Vehicle	0.95	0.38	0.78	1.32	2.25	0.22	1.51	3.93	0.69	1.20	2.26
60	0.99	0.51	0.58	1.08	2.76	0.21	1.50	3.37	0.57	0.98	1.85
120	0.90	0.29	0.50	0.77	2.77	0.16	1.07	2.75	0.47	0.80	1.68
SEM	0.13	0.066	0.15	0.15	0.54	0.025	0.27	0.59	0.084	0.13	0.33
P-value	0.90	0.097	0.42	0.05	0.74	0.18	0.44	0.40	0.20	0.14	0.44

[1]Values represent least squares means and pooled standard errors of the means with associated *P*-values for the effect of NPY treatment within diet and gene. Different superscipts within an effect for each gene are significantly different at $P < 0.05$, Tukey's test. Abbreviations: 1-acylglycerol-3-phosphate O-acyltransferase 2: AGPAT2; adipose triglyceride lipase: ATGL; diacylglycerol O-acyltransferase 2: DGAT2; CCAAT/enhancer-binding protein alpha and beta: C/EBPα and C/EBPβ, respectively; fatty acid binding protein 4: FABP4; neuropeptide Y: NPY; NPY receptor 2: NPYR2; lipoprotein lipase: LPL;

peroxisome proliferator-activated receptor gamma: PPARγ; sterol regulatory element-binding transcription factor 1: SREBP1.

Table 5-5b. Abdominal fat mRNA abundance at 3 h post-injection.

Effect[1]	AGPAT2	ATGL	DGAT2	C/EBPα	C/EBP$_\beta$	FABP4	NPY	NPYR2	LPL	PPARγ	SREBP1
HC Diet											
Vehicle	0.48	0.25	0.44	0.55	2.11	0.17	0.17	0.15	0.27	0.49	1.12
60	0.44	0.29	0.30	0.57	1.84	0.16	0.43	0.24	0.23	0.52	0.91
120	0.42	0.15	0.28	0.45	1.41	0.14	0.15	0.13	0.22	0.46	0.89
SEM	0.051	0.049	0.10	0.11	0.29	0.015	0.12	0.066	0.046	0.088	0.19
P-value	0.64	0.14	0.49	0.73	0.25	0.21	0.18	0.47	0.75	0.87	0.64
HF Diet											
Vehicle	0.45	0.19	0.30	0.45	1.91	0.13	0.44	0.11	0.22	0.54	2.48
60	0.59	0.24	0.31	0.53	2.36	0.16	0.49	0.18	0.26	0.62	2.04
120	0.45	0.13	0.38	0.50	2.06	0.12	0.29	0.16	0.23	0.50	2.18
SEM	0.071	0.043	0.098	0.085	0.43	0.015	0.14	0.028	0.051	0.12	0.64
P-value	0.32	0.24	0.83	0.82	0.77	0.16	0.60	0.26	0.90	0.77	0.89
HP Diet											
Vehicle	0.49	0.32	0.13	0.32	3.02	0.10	0.32	0.19	0.15	0.29	1.22
60	0.40	0.27	0.28	0.37	3.29	0.089	0.26	0.20	0.19	0.39	1.66
120	0.41	0.20	0.27	0.42	2.60	0.11	0.47	0.49	0.22	0.38	1.54
SEM	0.072	0.050	0.062	0.052	0.53	0.012	0.078	0.16	0.026	0.069	0.39
P-value	0.62	0.22	0.18	0.40	0.59	0.59	0.17	0.37	0.16	0.54	0.70

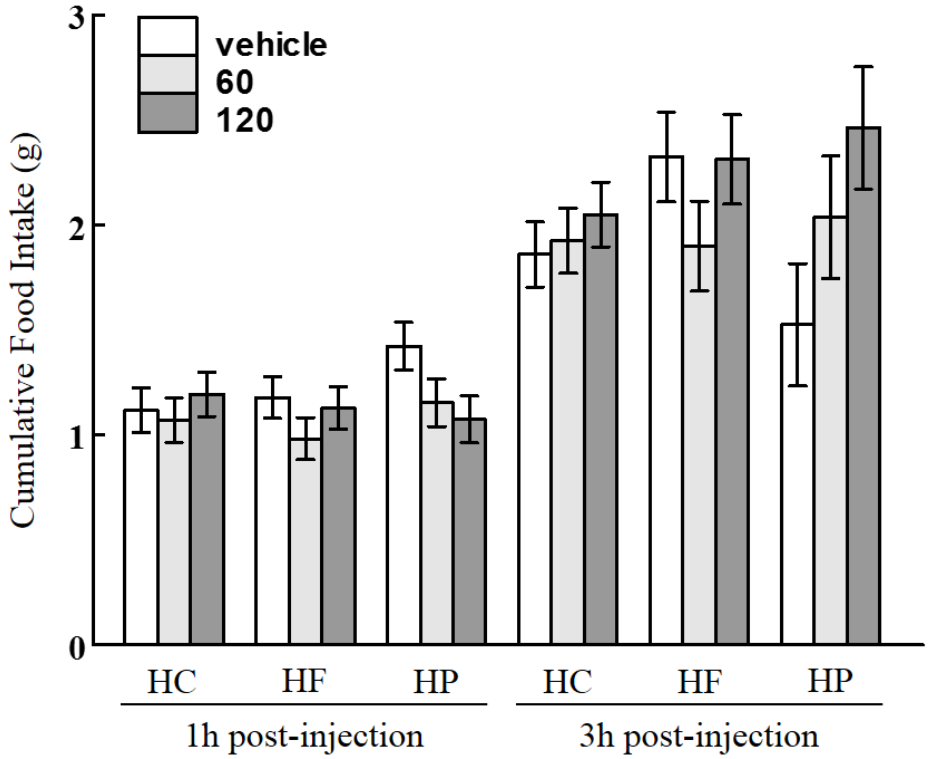

Figure 5-1. Food intake at 1 and 3 h post-injection. Chicks fed one of three diets (HC: high carbohydrate; HF: high fat; HP: high protein) were injected with vehicle, 60, or 120 µg/kg neuropeptide Y at 4 days post-hatch with n =10 per group. Data are means ± S.E.M.

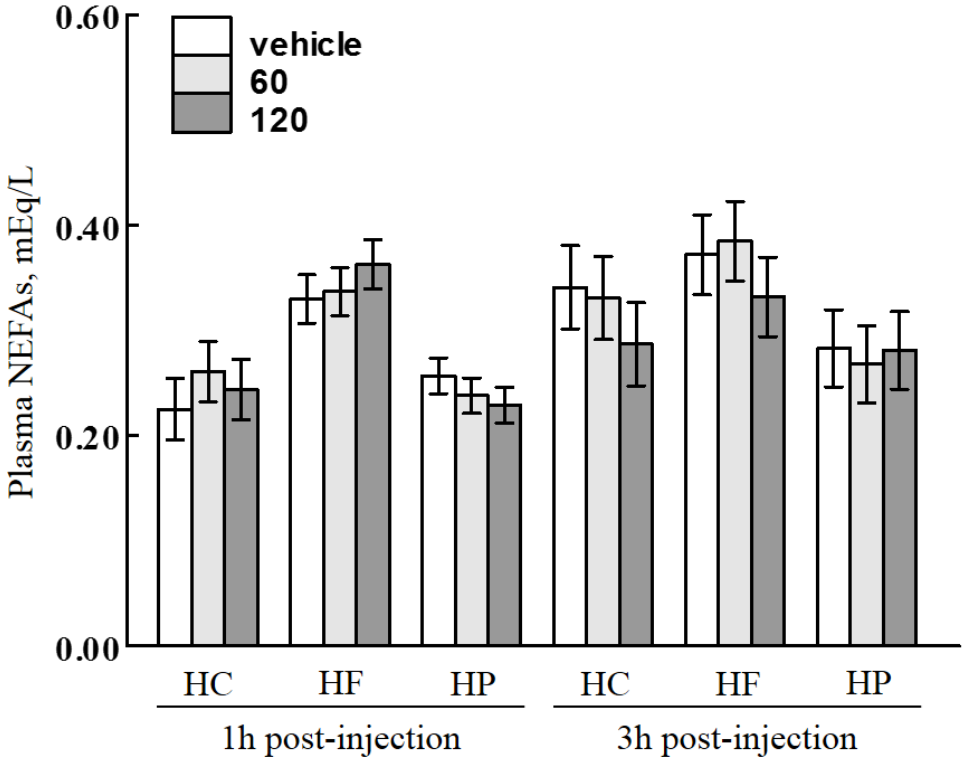

Figure 5-2. Plasma non-esterified fatty acids at 1 and 3 h post-injection. Chicks fed one of three diets (HC: high carbohydrate; HF: high fat; HP: high protein) were injected with vehicle, 60, or 120 µg/kg neuropeptide Y at 4 days post-hatch with n =10 per group. Data are means ± S.E.M.

Chapter 6: Epilogue

This dissertation consists of a series of experiments designed to determine how dietary macronutrient composition affects NPY's effect on adipose tissue development in chickens during the early post-hatch period. Most of our knowledge on adipose physiology is derived from rodent and human studies with less known in the avian species. Both mammalian and avians species can be precocial or altricial. For my research, the animal model used are chickens (altricial) compared to rodents and humans (precocial), therefore, adipose tissue physiology may be different. Thus, my research has helped contribute to our understanding of adipose tissue physiology in avian species.

Firstly, it should be noted that all of these experiments were conducted in concert with another dissertation project that focused on the appetite regulatory aspects of the diets (207). It is impossible to interpret the effects of the diets on adipose tissue development without first acknowledging that the diets might be consumed in different amounts and lead to activation of different central nervous system pathways that in turn regulate energy intake and metabolism in the body. In mammalian studies, HP diets tend to suppress appetite (248). Our preliminary study also showed that the HP diet decreased food intake relative to the HC and HF diets during the early post-hatch period in chicks. In general, there are a variety of explanations for why HP diets inhibit feeding, including palatability or induction of satiety (248). It is important to note that one important factor that influences dietary preference in mammals is the composition of the previous diet, including the concentration of protein and the protein to energy ratio (249).

We also found that feeding a HF diet influenced dietary preference and adiposity. In our previous studies where we evaluated the effect of high-fat diets on appetite regulation, 60% of the

metabolizable energy in the diet was derived from lard (250). When provided this HF diet during the first 4 days post-hatch, chicks consumed a similar amount as those fed the HC and HP diets (250). However, the body weights were much less than chicks from the HC and HP diet groups. When chicks had access to all three diets, they ate less of the HF than HC and HP diets (250). Additionally, the supplemental source of fat in the HC and HP diets was soybean oil rather than lard. Thus, soybean oil was used as the supplemental fat source in this dissertation research and we formulated the HF diet to have lower amounts of supplemented fat in order to prevent the impaired growth associated with too high a concentration of fat in the diet. During our preliminary studies (not reported in this dissertation), HF diets that were formulated to contain 15 or 30% of the metabolizable energy from soybean oil were fed to chicks without any adverse effect on body weight gain. Thus, the HF diet containing 30% metabolizable energy from soybean oil was used for all of the experiments reported in this dissertation.

However, chicks respond differently to a HF diet as compared to mammals. From an evolutionary standpoint, it is natural behavior for animals to prefer HF (and high sugar) diets and it is hypothesized that in humans this relates to the ability of energy-dense, highly-palatable nutrients to trigger hedonic aspects of food intake that enable the individual to accumulate energy reserves that can be used during future periods of famine (251). Specifically, HF diet consumption is known to activate dopaminergic neurons in the mouse brain, leading to behavioral changes and reinforcement of over-consumption (252). In mammalian studies, HF diets have been widely used to induce obesity and continue to be the gold standard for inducing a state of insulin resistance in adults. Chronic consumption of a HF diet might contribute to a dysregulated nutrient sensing network, changes in the composition of the gut microbiota,

changes in gut function and/or other physiological mechanisms that alter energy intake and energy storage and expenditure (253, 254).

Because our previous research (250) and preliminary data showed that chicks do not have a preference for HF diets, it is still unclear how the palatability of the diets used influenced their relative demand by the chicks. High fat diet feeding did not increase fat deposition in chicks during the early post-hatch period. In addition, the diameter of adipocyte was greater than in those fed the HC and HP diets. As I discussed in the literature review, we have a very limited understanding in terms of the physiological changes induced by HF diets in chickens or other avian species. However, we did show that a HF diet containing 30% of the metabolizable energy from soybean oil did not result in increased food intake during the first week post-hatch, whereas the increased energy availability from increased food intake of the HF diet in rodent studies might explain its obesogenic effect (255). It is important to note developmental differences between mammalian and avian studies. Most of the rodent diet studies were conducted after weaning in sexually mature animals (252, 253), whereas our studies were conducted during the first few days after hatch in the chick. As described earlier in the dissertation (page 11), this is a time during which the gastrointestinal tract is still structurally and functionally immature and the chick is transitioning metabolically. Thus, results may have differed considerably had we fed the diets to older chickens.

As discussed in the literature review, researchers have started to elucidate the mechanisms regulating adipose tissue development in avian species; however, the pathways remain poorly understood. According to mammalian studies, each adipose tissue depot may have distinct morphological features and biochemical activities, and those differences are regulated by a variety of factors, including dietary macronutrient composition (172). In mammals, visceral fat

(fat wrapped around major organs, including the liver, pancreas and kidneys) is associated with metabolic disorders, whereas subcutaneous fat is considered to be relatively benign (50, 51). However, little is known about whether such differences exist in avian species. In the previous avian studies, abdominal fat was the focus and mechanisms of dietary macronutrient composition on subcutaneous fat were not reported (7). In this dissertation, chapter 3 was the first report of how dietary macronutrient composition affects adipogenesis and lipid metabolism in the clavicular and subcutaneous fat. We concluded that transcriptional regulation of adipogenesis-associated factors might be altered in response to fasting in order to down-regulate preadipocyte differentiation and decrease adipocyte hypertrophy in a depot-dependent manner. Furthermore, dietary macronutrient composition also affects adipogenesis and lipid metabolism in a depot-specific manner, as reported in the mammalian studies (172).

One may criticize that our experiment lasted for only 4 days or until day 4 post-hatch, which may not have been enough time to capture developmental effects. For instance, we previously reported that the abdominal fat depot is the fastest to develop after hatch in broilers and that there is a dramatic increase in abdominal adipose tissue weight between day 4 and 14 post-hatch (174). As introduced in the first chapter, the early post-hatch period represents a large proportion of the market broiler's life. We hypothesize that in order to affect adipose tissue expansion later in life, we should study the early stages of development that involve greater amounts of hyperplasia. Because the different depots in chicks display such large differences in their relative expansion after hatch, comparing molecular features during this time may provide insights on the pathways that are largely responsible for adipose tissue development and expansion. In addition, day 4 is an age at which the yolk sac has been utilized and chicks are completely dependent on the diet for energy and nutrients. Our results clearly demonstrated that 4 days of feeding diets with

different macronutrient composition affected food intake, organ weights, adipocyte area and diameter, as well as the expression of genes that are associated with adipogenesis and lipid metabolism. Therefore, 4 days is enough time for manifestation of different physiological responses to dietary macronutrient composition. In addition, three-phase feeding is used in the broiler industry and includes the starter (first 1-2 weeks post-hatch), growing (3-4 weeks post-hatch) and finisher (5-6 weeks post-hatch) phases of production (256). Nutrient requirements differ during each feeding phase, with a gradual increase in energy requirements and decrease in crude protein requirements that reflect the growth of the broiler (256). Because of differences among nutrients in their relative digestibility that change as the bird matures, it is rather complicated to study effects of a single nutrient independent of others in the diet during development.

In the fasting state, plasma NEFAs are released almost entirely from the hydrolysis of TAGs in adipocytes (38). However, dietary fat consumption can contribute to plasma NEFAs, accounting for up to 40-50% of the plasma NEFA pool during the postprandial period in humans (257). To quantify lipolysis in the adipocyte, the amount of NEFAs released from the adipose tissue is measured. According to a human study, the main source of NEFAs from adipose tissue is from abdominal subcutaneous fat, whereas only a small proportion of systemic NEFAs are liberated from the intraabdominal fat depot (258). However, such variation in avian species is unreported. To determine the contribution of each adipose tissue depot to plasma NEFAs, isotope dilution/hepatic vein catheterization techniques were used in the above-mentioned human study (258); however, such techniques have not been adopted in avian research as far as I know, and might be a challenge to optimize.

One of the challenges in avian physiology research is the limited availability of antibodies that cross-react with avian proteins. In addition, phosphorylation is required to activate some enzymes. We measured the mRNA abundance of lipolysis-associated factors ATGL and PLN1 (40, 259), which provides some insights on how these gene products and associated pathways are regulated, but does not capture what is occurring at the protein and activity level. As discussed in the literature review, the liver is the main site of de novo lipogenesis in avian species and humans, whereas adipose tissue is the major site in pigs and rodents (19, 55). Thus, I did not measure in adipose tissue the expression of two central enzymes involved in de novo lipogenesis, acetyl-CoA carboxylase and fatty acid synthase (76). Instead, we measured the mRNA abundance of two important TAG synthesis-related enzymes AGPAT2 and DGAT2 (236, 260).

Another focus of this dissertation is the effect of NPY on adipose tissue development and how that is affected by dietary macronutrient composition. In the central nervous system, NPY has the highest concentration within the hypothalamus, anterior pituitary and brain stem. In addition, there is a wide distribution in the periphery, such as in the sympathetic nerves, platelets, and different cell types within the white adipose tissue (228). As a neurotransmitter co-stored with norepinephrine in the sympathetic nerve terminals, NPY can exert effects in the white adipose tissue through the neuroendocrine route (261). Furthermore, the effect of NPY can also be exerted via an autocrine pathway by mature adipocytes, a paracrine mechanism by immune cells, and endocrine pathway by platelets through the circulation, or across the blood-brain barrier (229, 262). Thus, we administered NPY centrally and peripherally to investigate the effect of both routes on adipogenesis and lipid metabolism, which is novel in avian species.

In chapter 4, we reported that central NPY injection increased the expression of adipogenesis-associated factors in the subcutaneous and abdominal fat of chicks that were fed the HF diet,

indicating that consuming more fat makes chicks more sensitive to the effect of NPY on stimulating adipogenesis. Similarly, we previously showed that chicks fed a HF diet were more sensitive to the effects of central NPY on increasing food intake (173). In addition, we revealed that ICV NPY injection decreased the expression of PPARγ and SREBP1 in the subcutaneous fat of chicks that were fed the HC diet. According to mammalian studies, both stress and a high fat and high sugar diet increase glucocorticoid signaling in the visceral fat, which in turn elevates the mRNA expression of NPY and NPYR2 in endothelial cells and adipocytes (91). We thus concluded that consumption of the HF diet might have sensitized the NPY system in chicks.

With intraperitoneal injection, NPY is absorbed into the mesenteric blood supply that is transported directly to the liver via the portal vein. Because this is not affected by degradation in the gastrointestinal tract, the bioavailability of intraperitoneally administered peptide is substantially high (263). However, in the liver, NPY might be biotransformed, hydrolyzed, partly stored to be released slowly and/or partly secreted into the bile. According to studies in humans, the half-life of NPY is up to 39 min (247). It is thus unclear how much NPY might diffuse between the central nervous system and periphery after injection. The blood-brain barrier of the 4 day-old chick may not be completely closed, therefore, NPY can enter the brain of mouse from blood by diffusion across the blood-brain barrier (244). Plasma NPY concentrations are reported to be 2 ng/mL in humans (264) and 5 ng/mL in rats (265), whereas NPY concentrations in the CSF are from 35 to 130 pg/mL in humans (266, 267) and 0.5 pg/mL in mice (268). In addition, there was 1 ng/mL of NPY in the plasma of chickens (269). However, the concentration of NPY in the CSF of avian species has not been reported to our knowledge. In chapter 4, ICV injection of 0.2 nmol NPY was performed, which is equivalent to 0.8 ng/mL (chicken NPY, M.W. 4246). This concentration is 5 to 22 times higher than the physiological concentration of NPY in human

CSF, therefore mimicking a pharmacological response if the concentration is similar in the CSF of chicks. However, this concentration is similar to the concentration of NPY in mouse CSF, thus mimicking a physiological response. In chapter 5, 60 µg/kg BW of NPY was IP injected in chicks on day 4 post-hatch. Because the injection volume was approximately 200 µL and BW of chicks was 80 g on average on the day of the experiment, the concentration of injected NPY was 24,000 ng/mL. This is a 4,800 and 12,000 fold-difference from the plasma NPY concentrations reported in rats and humans, respectively. Although circulating NPY is able to cross the blood-brain barrier, the concentration of NPY that entered the central nervous system might be lower than the threshold to increase food intake.

In chapter 5, we reported that food intake was not increased after peripheral NPY administration, suggesting that the effects of NPY on adipose tissue physiology were independent of secondary effects due to altered food intake. The expression of NPYR2 and NPY mRNAs increased in the subcutaneous fat of chicks fed the HP diet at 1 h after NPY injection and in the clavicular fat of chicks fed the HF diet 3 h after NPY injection, respectively. Thus, the injected NPY might have provided positive feedback for the increase in NPY and NPYR2 mRNA. Our previous *in vitro* studies showed that NPY treatment increased the mRNA expression of NPY and NPYR2 in the SVF of cells isolated from abdominal fat of 14 day-old broiler chickens at 4 h post-treatment, whereas the expression of NPY was decreased after NPY treatment in the same type of cells at 12 h post-treatment (95, 96). In addition, the mRNA abundance of NPYR2 was also increased in chicken adipose tissue-derived cells at day 2 and 6 post-induction, however, its expression remained unchanged at later days (95, 96). The stromal vascular fraction of adipose tissue is a source of different types of cells, including preadipocytes, mesenchymal stem cells, endothelial progenitor cells and adipose tissue macrophages (270). Taken together, the previous studies

showed that NPY also provided positive feedback to increase the expression of NPY and NPYR2 in the stromal vascular fraction of adipose tissue and adipocytes *in vitro*, though the effect might have disappeared at a later time point after NPY treatment.

In chapter 5, we reported that the effect of NPY on the up-regulation of some of the adipogenesis- and lipid metabolism-associated genes was more predominant in the clavicular and abdominal fat of chicks fed the HF or HP diet, suggesting a greater responsiveness of these depots and dietary groups to the adipogenic effects of NPY. Co-culture of preadipocytes/adipocytes and neurons using mouse 3T3-L1 preadipocytes/adipocytes also showed that an adipocyte-derived soluble factor upregulates the secretion of NPY in neurons dramatically, whereas neuron-derived NPY promotes the growth of adipocytes and endothelial cells (262). Furthermore, adipocytes and preadicpocytes can be affected by other cell types in the adipose tissue. Cells that originate outside of the adipose tissue, such as neurons, also might have an impact on the NPY concentrations in these cells. Due to the complicated interactions between preadipocytes/adipocytes and other cell types, it is unclear whether effects on adipose tissue were due to direct effects of circulating NPY that reached the adipose tissue after injection or indirect pathways activated by NPY in the brain or other tissues.

Downstream signaling pathways that are activated in response to NPY in adipose tissue have been identified in mammals, however, the intracellular mechanisms regulating lipid metabolism still remain poorly understood. Through the NPY receptor sub-type 1, there was activation of the extracellular signal-regulated kinase (ERK) ½ signaling pathway that is responsible for rodent preadipocyte proliferation (243). In addition, NPY promotes adipogenesis through the induction of the classic adipogenic genes, such as C/EBPα and PPARγ, via suppressing the cAMP response element-binding protein (CREB)-SIRT1 signaling pathway (271). As potent lipolytic

factors, catecholamines (especially norepinephrine) bind β-adrenergic receptors that activate the cAMP-protein kinase A signaling cascades (272). To inhibit lipolysis, NPY reduces CREB and SIRT protein expression via NPYR1 (271). However, it is still unknown whether such signaling pathways are responsible for NPY's effect or if different pathways exist in avian species.

Adipocyte development reflects a combination of hyperplasia and hypertrophy. We reported that HF diet consumption increased adipocyte diameter and that differences in adipocyte size exist in different adipose tissue depots in chickens. Because each cell contains the same amount of DNA, total DNA mass is directly proportional to the number of cells in the adipose tissue. Therefore, total DNA mass can be measured to indicate the change in hyperplastic growth from the adipose tissue. However, in addition to adipocytes, the SVF of the adipose tissue contains a variety of cell types, including preadipocytes, vascular endothelial cells, fibroblasts, and different immune cells. Thus, separation from the SVF will be necessary in order to focus on physiological changes that are specific to the adipocyte. Furthermore, it is known that other cell types besides adipocyte precursor cells and adipocytes produce NPY in adipose tissue. Thus, when we measure changes at the whole tissue level, we are not only possibly masking effects that are adipocyte-specific, but might also be measuring changes that can be attributed to other cell types. For instance, it has been reported that NPY can promote angiogenesis (273).

In conclusion, results from my research have contributed to the understanding of how diet composition and neuropeptide Y affect adipose tissue development in chicks. Reduced adipose tissue deposition in chicks fed the HP diet might be explained by decreased rates of adipogenesis and associated fatty acid incorporation into fats in the lipid droplet of the adipocyte. In contrast, more fat and larger adipocytes in chicks fed the HF diet likely result from greater rates of adipocyte hypertrophy. Consumption of the HF diet might have sensitized chicks to the effect of

central NPY injection on food intake and adipogenesis, which has implications in both animals and humans for understanding the effects of an appetite-regulatory peptide on energy balance. In light of the current obesity epidemic in humans and concerns about excess fat accumulation in agriculturally-important species, such information is particularly relevant and timely.

A logical continuation of this research would include experiments designed to elucidate the cellular signaling pathways responsible for effects of NPY on specific cell types in the adipose tissue, using a cell culture model. Experimental manipulations, including NPY receptor antagonism or gene suppression/deletion, should be explored to understand mechanisms regulating lipid metabolism and preadipocyte/adipocyte proliferation and differentiation. In addition, co-culture of chicken preadipocytes/adipocytes with neurons will also be a fruitful avenue to investigate the interactions between preadipocytes/adipocytes and neurons.

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