Molecular and cellular mechanisms of energy homeostasis in birds

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Abstract (Academic)

Hypothalamus and adipose tissue are essential central and peripheral sites regulating energy homeostasis. Disruption of energy homeostasis can lead to diseases like anorexia and obesity in humans and reduced productivity in animals. Therefore, integrating knowledge in hypothalamic appetite regulation and adipose tissue metabolism is essential to maintain homeostasis. The aim of this dissertation was to elucidate molecular and cellular mechanisms of energy homeostasis in birds. We determined adipose tissue physiological changes during the first two weeks post-hatch in chickens from lines selected for low (LWS) and high (HWS) body weight. LWS was more dependent on yolk and subcutaneous fat mobilization for growth from hatch to day 4 post-hatch, with hyperplasia-predominated replenishment of the reservoir. In contrast, HWS was more dependent on feed for growth and maintained depot mass through hyperplasia and hypertrophy.

From day 4 to 14 post-hatch, compared to maintenance of depot weight and adipocyte size in LWS, HWS accumulated clavicular and abdominal fat with minimal lipolysis. There was greater expression of precursor and proliferation markers in LWS with more apoptotic cells in the abdominal stromal vascular fraction on day 14 post-hatch, suggesting that apoptosis contributed to lower adipogenic potential and lack of abdominal fat in LWS. Exposure to thermal and nutritional stressors at hatch impaired growth by reducing yolk utilization and lowering body weight, lean and fat masses in LWS. Stress exposure resulted in increased global DNA methylation and DNA methyltransferase activity in the arcuate nucleus of the hypothalamus in LWS. Moreover, there was decreased binding to methyl-CpG-binding domain protein 2 in the promoter of corticotropin-releasing factor (CRF) because of hypomethylation in one CpG site at
its core binding site in stressed LWS, which explains the increased CRF expression in the paraventricular nucleus of the hypothalamus. We next determined effects of nutritional status on adipose tissue physiology in Japanese quail, a less-intensively selected avian species. Six-hour fasting promoted lipolysis and gene expression changes in 7-day old quail with some changes restored to original levels within 1 hour of refeeding. Overall, our results reveal novel cellular and molecular mechanisms regulating appetite and adiposity in birds early post-hatch.
Abstract (Public)

Hypothalamus and adipose tissue are essential for regulating energy homeostasis in central and peripheral body sites, respectively. Disruption of energy homeostasis can lead to diseases like anorexia and obesity in humans and reduced productivity in animals. Therefore, integrating knowledge in hypothalamic appetite regulation and adipose tissue metabolism is essential to maintain energy homeostasis in both humans and animals. The aim of this dissertation was to elucidate molecular and cellular mechanisms of energy homeostasis in birds. We first determined adipose tissue physiological changes in chickens during the first two weeks post-hatch from lines selected for low (LWS) and high (HWS) body weight. These chickens have been selected for juvenile body weight for over 60 generations. The LWS are lean and anorexic, while HWS eat compulsively and develop obesity and metabolic syndrome. Such characteristics make the body weight line chickens good animal models to study physiological changes under anorexia and obesity. We found that LWS was more dependent on yolk reserves and subcutaneous fat mobilization for growth from hatch to day 4 post-hatch, with replenishment of the fat reservoir by increases in cell number. By contrast, HWS was more dependent on feed for growth and maintained depot mass through increased cell number and cell size. From day 4 to 14 post-hatch, HWS accumulated fat throughout the body, with less fat breakdown as compared to LWS. There was greater expression of cellular precursor and proliferation markers in LWS, with more dying cells in their abdominal fat on day 14 post-hatch, suggesting that programmed cell death is responsible for the lack of fat cell development in LWS. Exposure to thermal and nutritional stressors at hatch impaired growth by reducing yolk utilization and lowering body weight, lean and fat masses in LWS. There were many molecular changes in the hypothalamus, including changes in DNA that led to increased activation of corticotropin-releasing factor (CRF), a
signaling molecule that is known to regulate the body’s stress and appetite responses. Stress exposure increased global DNA methylation and DNA methyltransferase activity in the arcuate nucleus of the hypothalamus in LWS. Moreover, there was less methylation at the core binding site of methyl-CpG-binding domain protein 2 (MBD2), a protein that binds to methylated DNA to repress gene expression, in the CRF gene, in stressed LWS. In response to stress, there was decreased binding of MBD2 to the promoter region of CRF, which may explain increased expression of CRF in the paraventricular nucleus of LWS. These results demonstrate that early-life stressful events can cause epigenetic changes (like DNA methylation) that lead to alterations in physiology and behavior that persist to later in life. We next determined effects of nutritional status on adipose tissue physiology in Japanese quail, which have undergone less artificial selection than chickens and are more representative of a wilder-type bird. Six-hour fasting promoted lipolysis and gene expression changes in 7-day old quail with some changes restored to original levels within 1 hour of refeeding. Overall, our results provide novel perspectives on cellular and molecular mechanisms regulating appetite and adiposity in birds during early post-hatch development.
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Chapter 1: Introduction

Energy homeostasis refers to the balance between energy intake and expenditure. When energy expenditure is not fully covered by energy intake, for instance, in anorexia nervosa (AN), there is a negative energy balance characterized by weight loss and decreased fat mass. In turn, if energy intake exceeds the expenditure, for instance, under the obese state, a positive energy balance is achieved, and the excess energy is stored in the adipose tissue [1-5]. In addition to being the primary energy storage site, adipose tissue is also one of the largest endocrine organs in the body. Hence, lack or excessive accumulation of adipose tissue can disrupt the endocrine system and affect physiological processes such as eating, growth, reproduction and the stress response [6]. Though the associations between fat mass and alterations in physiology are known to exist under AN and obese states, previous research on adipose tissue metabolism mainly focused on the visceral depot, which is thought to be closely correlated with the onset of metabolic diseases. Little is known about the metabolism in other adipose depots under either AN or obese states.

Anorexia nervosa is most prevalent in adolescents and young adults. Nonetheless, childhood (before puberty) onset of AN is an increasing problem [7]. Childhood AN has distinguishing symptoms that are not observed in adolescent or adult AN. Because children before puberty undergo rapid growth and development, symptoms reversible with weight restoration in anorexic adults may not be reversible in children [8]. The global prevalence of childhood obesity (under 5 years old) has also increased from 4.2% in 1990 to 6.7% in 2010, and is predicted to reach 9.1% in 2020 [9]. It was reported by the World Health Organization (WHO) that in 2016, 41 million children under 5 years old were overweight or obese; over
340 million children and adolescents between 5 and 19 years old were overweight or obese; and more than 650 million adults over 18 years old were obese [5]. Growing evidence suggests that childhood obesity increases the risk of being obese in adulthood, the development of chronic diseases, and mortality and morbidity [9]. Therefore, it is important to understand the pathogenesis of childhood AN and obesity and identify effective and side effect-free strategies to restore energy homeostasis in order to prevent long-term detrimental effects on health.

Both genetics and epigenetics contribute to the pathogenesis of AN and obesity. Early-life stress exposure is a critical environmental factor that promotes epigenetic changes in diverse organs, including the hypothalamus, the key site responsible for the regulation of energy homeostasis [10]. Nuclei involved in appetite regulation and the stress response are located within the hypothalamus. Signaling interruption in the hypothalamus upon stress exposure can sequentially alter appetite regulation and thus affect adiposity [11-13]. Human population studies have revealed correlations between early-life stress exposure and epigenetic modifications in the hypothalamus, and associations with AN and obesity in adulthood. Animal models are needed to further elucidate the molecular mechanisms of early-life stress-induced alterations in appetite regulation and adiposity.

In this dissertation, Chapter 2 provides a comprehensive literature review of adipose tissue physiology under anorexic and obese states and the metabolic changes related to stress-induced epigenetic modifications in appetite regulation and energy metabolism. Further discussion is focused on the current knowledge gap based on human research and rodent models. The main aim of this dissertation, as described in Chapters 3, 4, and 5, was to use body weight-selected line chickens as anorexic and obese models to elucidate the cellular and
molecular differences in depot-specific adipose tissue physiology during early development and reveal mechanisms of hypothalamic appetite regulation in response to early-life stress exposure. The use of this chicken model may shed light on the mechanism of adipose tissue expansion that underlies a propensity to be lean or obese in humans due to the anatomical similarities between the two species. This research also provides insights in understanding the molecular basis for eating disorders and identifying novel strategies to alleviated stress-induced changes in eating behavior. Additionally, in Chapter 6, we used an alternative avian model, the Japanese quail, to understand how nutritional status (fed, fasted, and refeed state) alters adipose tissue physiology in the short-term during the early post-hatch period. These results suggest that adipose tissue physiology is very dynamic and influenced by a multitude of factors, including short-term food withdrawal and refeeding. Lastly, in Chapter 7, the epilogue, I discuss the implications of our findings and suggested directions for future research.
References

Chapter 2: Literature review

Stress and adipose tissue physiology under anorexic and obese states

1. Adipose tissue physiology

Adipose tissue is the primary organ that stores energy in the body and is the largest endocrine organ, regulating the secretion of diverse hormones and cytokines that control physiological changes in energy metabolism, thermoregulation, and glucose homeostasis, as well as psychosocial imprints [1-3].

The development and remodeling of adipose tissue are dynamic processes that continue throughout life. There are two main classes of adipose tissue—white adipose tissue (WAT), which is mainly composed of white adipocytes that are designed for energy storage, and brown adipose tissue (BAT), which is mainly comprised of brown adipocytes and largely functions to dissipate energy in the form of heat during non-shivering thermogenesis [4]. A third type of adipocyte—beige (also called brite/inducible BAT), was found to arise in WAT in response to various cues such as chronic cold exposure and sympathomimetic treatments, with thermoregulatory and energy balance functions [5].

WAT is distributed throughout the body mainly in subcutaneous (sWAT), visceral (vWAT), inter- (itMAT) and intramuscular (iMAT) as well as bone marrow (MAT) depots [6], among which sWAT and vWAT are of the most metabolic importance. In rodents [7] and chickens [8, 9], sWAT appears prior to vWAT during embryonic development. Subcutaneous WAT is located underneath the skin, inside the abdominal cavity, and within the skeletal muscles, which provides insulation from heat or cold [10]. In humans, about 80% of body adipose tissue is sWAT, with
the main depots being abdominal, (upper back) subscapular, gluteal and thigh, while vWAT contributes to 6 – 20% of total body fat [11]. Visceral WAT is distributed inside the peritoneum, and around internal organs (e.g., stomach, liver, intestines, and kidneys), which protects the tissue as padding [10]. Visceral WAT can be sub-divided into mesenteric, retroperitoneal, perigonadal and omental adipose tissue in accordance with its location [10]. Increased vWAT accumulation is positively associated with the onset of metabolic diseases [12], whereas increased sWAT is more benign and has little association with the development of metabolic disorders [13].

1.1 Adipose tissue development

Adipocytes are derived from either the embryonic mesoderm germ line [14], or from locations outside of the mesoderm, such as the neural crest [15]. Once formed, adipocytes occupy about half of the total cell number in adipose tissue [16], with other cell types including adipocyte precursors at various stages of differentiation, blood cells, endothelial cells, pericytes, and immune cells [17].

The stromal vascular fraction (SVF) is a part of adipose tissue that contains a heterogeneous mixture of cells, including mesenchymal stem cells, fibroblasts, epithelial cells, endothelial cells, hematopoietic cells, neural cells, macrophages, leukocytes, and T regulatory cells [18]. In humans, SVF cells from sWAT are more proliferative and adipogenic and less apoptotic than those from vWAT [19-21].

Mesenchymal stem cells (MSC) refer to the fibroblast-like, non-hematopoietic fraction of cells [22], which have the potential to differentiate into myoblasts, chondroblasts, osteoblasts, fibroblasts or adipocytes under certain induction conditions [23]. Adipogenesis refers
collectively to the sequential processes involved in the differentiation of a MSC to an adipocyte. MSC were first isolated from bone marrow by Friedenstein et al. in 1970 [24]. Later, it was discovered that MSC could also be acquired through liposuction aspiration of adipose tissue, where the SVF contained more stem cells (0.4 – 12 %) [25-27] than those derived from bone marrow (0.001 – 0.01 %) [28]. After the discovery of the immunological characteristics of MSC, the criteria for classifying a cell as a MSC have been defined by the International Society for Cellular Therapy as the following: 1) can adhere to plastic under standard culture conditions; 2) over 95% of the cells should express surface specific antigens CD73, CD90 and CD105, while lacking the surface antigens CD45, CD34, CD14, CD11b, CD19, CD79a and human leukocyte antigen (HLA) class II; and 3) can differentiate into multiple lineages under certain conditions in vitro [29].

Differentiation of a MSC involves its commitment to a specific lineage of progenitor cells, such as preadipocytes for the adipocyte lineage. The commitment to preadipocytes is regulated at the transcriptional level with no requirement for the presence of hormones [17]. Multiple pathways are involved in the regulation of the commitment procedure, which are well characterized and reviewed by Chen, Shou [30]. Preadipocytes have a fibroblast-like morphology and express preadipocyte factor 1 (Pref-1) (also known as delta-like homolog 1 [DLK1]), which is absent in adipocytes [31]. Therefore, Pref-1 is the only broadly used marker to distinguish preadipocytes from other cell stages during differentiation [14]. Pref-1 was first cloned from mouse 3T3-L1 preadipocytes [32]. It plays an inhibitory role in adipogenesis. The cleavage of Pref-1 (A and B isoforms) by tumor necrosis factor-α (TNFα) converting enzyme produces 50 kDa soluble forms of Pref-1, which sequentially target extracellular signal-regulated kinase (ERK; also known as mitogen-activated protein kinase [MAPK]) [33]. Phosphorylation of ERK1/2 (for chickens, only
ERK2 [34]) subsequently upregulates the expression of SOX9, which is critical for chondrogenesis. Upon the binding of SOX9 to the promotor regions of CCAAT-enhancer-binding proteins β (C/EBPβ) and δ (C/EBPδ), adipogenic activity is suppressed [35]. Meanwhile, membrane-bound, insoluble Pref-1 was found to inhibit preadipocyte proliferation by blocking cell transition from the G1 to S phase [36]—the time when C/EBPβ is highly phosphorylated and active [37]. These together suggest a critical role of Pref-1 in preadipocyte proliferation and differentiation.

Following the commitment of a MSC to a preadipocyte, terminal differentiation proceeds and includes cellular maturation into adipocytes with characteristic morphological changes from a fibroblast-like shape to a spherical shape [38]. The morphological change is induced by the change in composition of the extra-cellular matrix components, such as α-tubulin and β-actin [39]. A large number of extracellular factors are involved in the regulation of adipogenesis, including insulin, growth hormone, retinoids, thyroid hormone, glucocorticoids, and so on [4, 40, 41]. The detailed mechanisms were nicely reviewed in [42] and [37]. Over 100 transcription factors are involved in adipocyte development, among which peroxisome proliferator-activated receptor γ (PPARγ), C/EBPs, sterol regulatory element-binding proteins (SREBPs) and Krüppel-like factors (KLFs) are the most important and well-studied [40].

Upon hormonal induction, increased cyclic adenosine monophosphate (cAMP) transiently (< 4 hours) stimulates the expression of C/EBPδ and C/EBPβ—the transcriptional activators of adipocyte differentiation, through phosphorylating cAMP response element-binding protein (CREB). C/EBPβ sequentially activates PPARγ and C/EBPα by binding to regulatory elements in their respective proximal promotors [41]. C/EBPα and PPARγ cross-activate each other.
through positive feedback and C/EBPα maintains their expression during differentiation [43] (Figure 2.1).

The activation of C/EBPβ and C/EBPδ also induces the expression of KLF5, which belongs to a large zinc-finger protein family that regulates apoptosis [40], and the expression of SREBP1c, which belongs to basic helix-loop-helix-leucine zipper protein family [44]. KLF5 promotes the activation of PPARγ through promoter binding [45], while SREBP1c activates PPARγ through an endogenous fatty acid derivative ligand [46]. PPARγ is considered sufficient to activate the progression of adipogenesis and has long been regarded as the master regulator of this process. However, a recent study revealed that SREBP1a expression is stimulated within an hour post-induction, prior to the increased expression of PPARγ (6-7 hours post induction) and C/EBPα (14-15 hours post-induction). Moreover, knockdown of SREBP1a mRNA significantly reduced the expression of PPARγ and C/EBPα, which suggests that SREBP1a is an upstream regulator of PPARγ and C/EBPα that initiates adipocyte differentiation [46]. The specific mechanisms involved have not been reported. Activated C/EBPα and PPARγ induce a cascade of transcriptional activation of downstream target genes, with some encoding enzymes that are involved directly or indirectly in lipid metabolism. Examples include fatty acid binding protein 4 (FABP4, also known as adipocyte protein 2 [aP2]), which mediates the transportation and uptake of long-chain fatty acids and serves as a marker of intermediate differentiation [47], and glycerol-3-phosphate dehydrogenase, which contributes to triacylglycerol (TAG) synthesis and serves as a marker of late differentiation [48]. These downstream factors facilitate the maturation of adipocytes, which involves accumulation of lipids, and termination of the differentiation process.
Adipogenesis is regulated by not only the afore-mentioned pro-adipogenic factors, but also repressors that suppress differentiation (Figure 2.1). For instance, KLF 2 and KLF7 exert their anti-adipogenic effects by inhibiting promotor activation of PPARγ and C/EBPα genes, respectively, thereby suppressing the downstream differentiation process [49, 50]. C/EBP homologous protein suppresses adipogenesis by inhibiting the transcription of both PPARγ and C/EBPα during the first 2-4 days post-induction of differentiation [51]. GATA binding proteins 2 and 3, DNA binding proteins that are involved in cell proliferation and maturation through transcriptional regulation, inhibit both PPARγ and C/EBPα through protein-protein interactions [52]. Their stimulation during preadipocyte proliferation and downregulation during adipocyte differentiation suggest that they play an important role as preadipocyte factors.

1.2 Molecular regulation of lipid synthesis and lipolysis

1.2.1 Lipid synthesis

1.2.1.1 Triglyceride synthesis through uptake of circulating fatty acids

Triglycerides are stored in adipocytes and represent the largest energy reserve in the body [53]. TAG deposition upon the consumption of a eucaloric diet (diet to maintain body weight) is mediated through the lipoprotein lipase (LPL) pathway [54]. LPL is mainly synthesized in myocytes, adipocytes, and macrophages, which is then transported by glycosylphosphatidylinositol anchored high density lipoprotein binding protein 1 into the luminal surface of capillary endothelial cells [55]. The catalytically active form is predominant in adipose tissue during the fed state, whereas the catalytically inactive form is predominant during the fasting state [56]. During TAG synthesis, three non-esterified fatty acids (NEFA) are esterified to one glycerol backbone. NEFA are acquired either from the breakdown of dietary fat in the form
of chylomicrons through LPL catalysis, or from circulating TAGs carried by very low density lipoprotein (VLDL), which is hydrolyzed by LPL to release fatty acids and glycerol [57]. In chickens, less than 5% of dietary fat (based on a standard diet, in the form of portomicrons, the analogous form of chylomicrons) contributes to TAG synthesis [58]. Hence, TAG deposition is predominantly dependent on VLDL transport in chickens. Uptake of NEFA from extracellular pools is mediated by fatty acid transport proteins or fatty acid translocases (also known as CD36) [59].

In adipose tissue, due to the lack of glycerol kinase, glycerol-3-phosphate (G3P) is acquired mainly through glycolysis, which is induced by insulin. In liver, because the expression of glycerol kinase is high, G3P can be acquired directly through plasma glycerol [60]. In diabetic patients, glyceroneogenesis, a pathway that synthesizes G3P through non-carbohydrate substrates, is the major contributor of G3P [61].

Upon the catalysis of fatty acyl-CoA synthetase, NEFAs are activated into NEFA-CoA. Glycerol-3-phosphate acyltransferase (GPAT) and acyl-CoA acylglycerol-3-phosphate acyltransferases acylate NEFA-CoA and G3P into phosphatidic acid, which is then phosphorylated into diacylglycerols (DAG). TAG is subsequently formed via the catalysis of diacylglycerol acyltransferase (DGAT) [60] (Figure 2.2). Enzymatic activities of GPAT and DGAT are enhanced after treatment with a PPARγ agonist, which leads to an increased synthesis of TAG in ob/ob mice WAT [62]. These results indicate that PPARγ regulates adipocyte differentiation through stimulating the formation of TAG.
1.2.1.2 De novo lipogenesis

De novo lipogenesis (DNL) is a pathway that converts excess non-lipid precursor nutrients into fatty acids for energy storage. It takes place in both the liver and adipose tissue. In humans and chickens, 90% of DNL occurs in the liver, whereas in rodents, liver and adipose tissue contribute equally to DNL [63, 64].

Upon uptake by glucose transporter 2 into the liver, glucose is converted into pyruvate through glycolysis, which then enters the mitochondria and is oxidized into acetyl-CoA and condenses with oxaloacetate into citrate to enter the citric acid cycle. Citrate can exit the mitochondria and be converted into acetyl-CoA in the cytoplasm by ATP citrate lyase, which links the metabolism between carbohydrates and fatty acids. Acetyl-CoA carboxylase subsequently catalyzes the conversion of acetyl-CoA into malonyl-CoA, which then serves as substrate for the sequential addition of carbons from acetyl-CoA to form palmitate via the catalytic actions of fatty acid synthase. Palmitate is further elongated by very long chain fatty acid elongase 6 into stearate and then desaturated by sterol-CoA desaturase 1 (SCD1) [65, 66]. SREBP1c and carbohydrate response element binding protein (ChREBP) play a crucial role in regulating these genes during DNL, where SREBP1c is predominantly in charge of hepatic DNL regulation, and ChREBP mainly regulates DNL in adipose tissue [60] (Figure 2.2).

Consumption of high-fat diets increases the expression of transcriptional coactivator peroxisome proliferator-activated receptor γ coactivator-1β (PGC-1β) [67]. PGC-1β is a coactivator of SREBP, which is a master regulator of lipogenesis, and liver X receptor, which regulates lipoprotein transport [68]. Overexpression and knockdown studies confirmed the role of PGC-1β in regulating the enzymes involved in hepatic DNL [67, 69].
1.2.2 Lipolysis

In humans, although sWAT is more abundant than vWAT and contributes the most to plasma NEFA, vWAT is more lipolytic than sWAT [54, 60]. White adipose tissue is innervated by the sympathetic nervous system, which triggers lipolysis in adipose tissue through signaling mediated after activation of β-adrenergic receptor subtypes 1-3 (β-AR1-3) [70]. Upon agonist binding to β-AR1-3, a cascade of factors are stimulated, including adenylyl cyclase, which controls cAMP production [71]. Intracellular elevation of cAMP subsequently activates protein kinase A (PKA), which phosphorylates hormone-sensitive lipase (HSL), and perilipin A, a protein that participates in lipolysis [70]. Adipose triglyceride lipase (ATGL), HSL, and monoglyceride lipase (MGL) are the three most important enzymes in lipolysis.

Adipose triglyceride lipase initiates lipolysis by hydrolyzing TAG to DAG with the concomitant release of one NEFA. The full catalytic activity is achieved by its co-activator comparative gene identification-58 (CGI-58) [60]. CGI-58 is localized on lipid droplets, which are coated by perilipin A [72]. In vitro experiments showed that the binding of CGI-58 on perilipin A-coated lipid droplets is dependent on adipocyte metabolic status, which is reflected by intracellular cAMP levels [73]. As mentioned, increased cAMP drives the phosphorylation of perilipin A, which facilitates the lipase to attack lipid droplets and the translocation of HSL to lipid droplets, hence accelerating lipolytic activity [74, 75]. HSL can catabolize both TAG and DAG, although it hydrolyzes DAG more efficiently [76]. Once DAG is hydrolyzed, another NEFA is released together with monoacylglycerol, which is hydrolyzed by MGL to release the last NEFA together with the glycerol backbone [76] (Figure 2.2).
An anti-lipolytic response is triggered through activation of α2-AR, which inhibits adenylyl cyclase activity and thus reduces cAMP production and subsequently inhibits the activation of the PKA pathway, which decreases the phosphorylation of HSL and perilipin A [70]. Upon feeding, an elevation in blood glucose triggers the release of insulin from pancreatic beta cells into the blood, one of its targets being its receptors on adipocytes in adipose tissue. Insulin receptor-mediated signaling triggers activation of phosphatidylinositol-3-OH kinase, which then acts to phosphorylate protein kinase B (PKB, also known as Akt), which then converts cAMP to the inactive form 5’ AMP through the catalysis of cGMP-inhibited phosphodiesterase-3B [77].

1.2.3 Adipose tissue expansion

The balance between fat deposition-associated pathways such as adipogenesis and hypertrophy and catabolic pathways such as lipolysis determines the degree of fat accumulation in a fat depot [78]. There are two cellular mechanisms for adipose tissue expansion: hypertrophy and hyperplasia. Hypertrophy is characterized by enhanced triglyceride storage and associated expansion of existing adipocytes, while hyperplasia refers to the increase in adipocyte cell number. Hypertrophy is predominant in adulthood obesity as it is observed in all overweight and obese individuals [42], whereas hyperplasia is a mark of the severity of obesity [37]. In both humans [79] and chickens [80], WAT emerges during embryonic development predominated by hyperplasia followed by hypertrophy. Adipose tissue expands rapidly after birth [38] while chickens have increased lipid uptake prior to hatch [80]. From embryonic day 12, the embryo rapidly accumulates lipid from the yolk, leading to the development of subcutaneous adipose tissue and increased TAG in the abdominal and thoracic depots [81]. During the hatching period (embryonic day 19 - 22), the embryo takes up 20 – 30 % of the total yolk lipid with no further TAG accumulation [81]. Abdominal fat mass increases rapidly during the first two weeks post-
hatch, and exceeds the weight of the subcutaneous fat pad on day 14, whereas the adipocyte cell size is greater in the subcutaneous compared to abdominal fat pad [82]. Factors such as genetic background, hormonal exposure, and diet can all affect the degree of hypertrophy and hyperplasia [4]. A study using obese-resistant and obese-prone mice illustrated that cell size change depends on diet, and cell number change depends on genetics and diet, as well as their interactions [83].

2. Adipose tissue physiology under anorexic state

2.1 Fat mass and adipose tissue distribution

According to the Diagnostic and Statistical Manual of Mental Disorders, anorexia nervosa (AN) is defined as a restricted energy intake relative to the requirement due to the intense fear of weight gain even at a significantly low weight, where the BMI is below 18 in adults and the corresponding BMI percentile in children and adolescents [84]. A meta-analysis of 62 studies published from 1996 to 2019 showed that female AN patients had 50% less body fat mass than their healthy counterparts on average, with fat being stored mainly in the trunk region [85]. Body fat loss has been observed in different age groups. Adolescent girls with AN had about 1/3 of the total body fat mass (4.1 ± 2.8 kg vs. 12.1 ± 2.9 kg) and half of the body fat mass as a percentage of body weight (10.1 ± 5.9 % vs. 23.1 ± 3.8 %) compared to age-matched healthy controls [86]. In adult women with AN, total body fat mass (4.0 ± 3.0 kg) and body fat percentage (9.8 ± 6.6 %) were also lower than their age-matched healthy controls (11.8 ± 1.8 kg and 23.0 ± 3.6 %, respectively) [86]. Interestingly, both adolescent girls and adult women with AN had lower percentages of extremity fat (adolescent 50.0 ± 6.7 % vs. 57.4 ± 3.3 %; adult 48.8 ± 10.2 % vs. 57.8 ± 4.5 %) but higher percentages of trunk fat (adolescent 40.5 ± 5.5 % vs. 35.6 ± 3.4 %; adult
compared to their respective healthy counterparts, indicative of fat redistribution with a preferred loss of extremity fat under the AN state in females [86]. However, the higher percentage of trunk fat observed here is contradictory to previous reports that the percentage of trunk fat was lower in adolescent girls [87] and not different in adult women [88] from their respective age-matched healthy controls. It should be noted that subjects recruited in these studies represented a range of ages and duration of AN, as well as menarche and menstruation status, which may all contribute to differences in fat redistribution. Similar to the observations in females, adolescent boys with AN had lower fat mass (6.7 ± 0.5 kg), percentage fat mass (12.8 ± 0.8 %) and percentage extremity fat (49.9 ± 1.6 % of total fat mass) than age-matched healthy controls (9.7 ± 0.7 kg, 15.8 ± 1.1 % and 55.5 ± 0.9 %, respectively), while the percentage of trunk fat was comparable to the controls (35.4 ± 1.2 % vs. 33.5 ± 0.9 %) [89]. In adult men, percentage trunk fat was higher in AN than in age-matched healthy subjects (48.8 ± 4.0 % vs. 44.3 ± 4.8 %) while percentage leg fat was comparable between the two groups [90]. Other studies on body fat distribution in the AN state, ranging from 1989 to 2014, were comprehensively reviewed in [91], including 7 studies that were conducted in adolescents (1 on male and 6 on female subjects), and 13 that focused on adult AN (all on female subjects). The main findings from the review were that: 1) Adolescent girls with AN experienced a significant loss of trunk fat while their loss of extremity fat as a percentage of body weight varied; 2) Adolescent boys with AN, in contrast to the girls, had a higher percentage of trunk fat than healthy controls but a preferential loss of fat in the extremities, which was hypothesized to be related to lower testosterone levels; 3) Adult females with AN had similar waist to hip or android (trunk and upper body) to gynoid (around the hips, breasts and thighs) ratios as healthy controls, but tended to lose more extremity fat than trunk fat in the AN state.
Differences in BAT were also detected in AN patients. One study reported that adult female AN patients displayed no detectable BAT activity under 22-24 °C before or after weight regain but a lower resting metabolic rate compared to the healthy counterparts [92]. Under cold exposure, only 1 out of 5 female AN patients and 2 out of 5 weight regained AN patients showed BAT activity, while BAT activity was detectable in 4 out of 5 healthy controls [93]. These results collectively suggest that there is an adaptive reduction in resting metabolic rate in AN patients to compensate for chronic fuel deficiency caused by restrictive eating behavior.

2.2 Depot-specific adipose tissue physiology

While substantial loss of adipose tissue in the trunk and extremity regions was reported in AN patients across different age groups, only a few studies distinguished between the loss of sWAT versus vWAT in the trunk region. In adult female AN subjects, the loss of vWAT contributed to less than 20%, while the sWAT contributed to over 80% of the total loss of abdominal adipose tissue [94, 95]. In line with these results, a study of 14 adult female AN patients showed that the cross-sectional area of vWAT was about 54% of the healthy controls while that of sWAT was only about 49% [96], indicating a greater loss of sWAT than vWAT in the AN state, similar to the observations reviewed in [91]. These studies also showed an average of about 50% loss of thigh fat in the female AN subjects. Moreover, adipocyte area of abdominal sWAT in adult female AN subjects was significantly smaller than in healthy counterparts [97]. Fat attenuation—an indirect measurement of tissue quality and composition, was also higher in abdominal sWAT, vWAT, as well as thigh fat and iMAT of adult AN women compared to the age-matched healthy controls, reflective of smaller adipocytes with reduced lipid content in these depots, which is a marker of systemic fibrosis [98].
Although research on abdominal and thigh fat in AN is limited, there is considerable focus on the MAT. In contrast to the reduced abdominal sWAT and vWAT as well as thigh fat and iMAT, there was increased MAT in the lumbar [94, 99, 100], femur [94] and tibia [99] in adolescent girls [99, 101] and adult women [94, 96], as well as in the iliac crest of an adult male [102] with AN. There was an inverse correlation between MAT/fat content in the lumbar and femur with body mass index (BMI) and thigh fat mass, and femur MAT was also inversely correlated with abdominal total adipose tissue and sWAT masses [94].

2.3 Endocrine disruption in AN

Serving as the largest endocrine organ in the body, WAT secretes a wide range of adipokines, including hormones (e.g. leptin, adiponectin, resistin and glucocorticoids) and pro-inflammatory factors (e.g. TNFα and interleukin 6 [IL-6]), and expresses insulin receptor, leptin receptor, steroid hormone (e.g. glucocorticoids, estrogen and androgen) receptors, thyroid hormone receptor and catecholamine receptors, etc. (reviewed in [2]). In AN patients, food intake is substantially reduced. Decreased food consumption triggers the release of ghrelin—an orexigenic peptide secreted from the stomach, which acts on the growth hormone secretagogue receptor 1a in the hypothalamus and triggers increases in the production of orexigenic peptides agouti-related peptide (AgRP) and neuropeptide Y (NPY) in neurons in the arcuate nucleus (ARC), to promote food intake [103] (Figure 2.3). Meanwhile, ghrelin also acts on the somatotrophs in the pituitary to stimulate the release of growth hormone (GH) [104]. While ghrelin promotes lipogenesis and inhibits lipolysis [105], GH inhibits lipid accumulation and potentiates lipid mobilization [106]. GH sequentially increases the production of insulin-like growth factor 1 (IGF-1)—a factor highly expressed in liver and adipose tissue [106], whereas its function in adipogenesis is controversial [106]. Ghrelin also plays a critical role in maintaining
glucose homeostasis under the fasting state. Uncoupling protein 2 (UCP2), which decreases ATP production and thus the ATP to ADP ratio, is upregulated by ghrelin to inhibit the secretion of insulin. On the other hand, ghrelin increases hepatic glucose output by stimulating gluconeogenesis through the activation of the GH pathway [105]. Elevated ghrelin and GH levels, together with hypoinsulinemia, were observed in adult female AN patients. However, hepatic GH receptor expression and binding capacity were low, leading to GH resistance and consequently, reduced expression of IGF-1 [107, 108], which is also observed in adolescent girls [87]. In turn, the low level of IGF-1 provides weak negative feedback on GH secretion, which may help maintain the high level of GH expression, and thus potentiates lipolytic activity to provide substrates for gluconeogenesis and maintain euglycemia [108]. Surprisingly, weight recovery in AN patients was associated with lowered glucose uptake than in both healthy and no weight recovery controls, which is indicative of decreased insulin sensitivity. This is postulated to be attributed to the loss of sWAT during the early stage of AN, which may impair lipid storage and restoration capabilities in the depot and thus divert lipid storage mainly to vWAT or other tissues (e.g. muscle and liver), eventually leading to insulin resistance [109].

Leptin is an adipose-derived cytokine that is secreted in proportion to body fat mass [2]. With the reduction in body fat mass under the AN state, serum/plasma leptin levels decreased in both adolescent girls [87] and adult women [97, 110]. Although not specifically studied in male AN patients, 72 hours of fasting in healthy lean men led to reduced serum leptin and IGF-1 concentrations, whereas daily administration of replacement doses of leptin restored IGF-1 levels to those at the initial day of the experiment [111], demonstrating the interaction between leptin and IGF-1 during energy balance regulation. Indeed, leptin and IGF-1 expression in adipose tissue are positively correlated whereas an antagonistic effect between leptin and IGF-1 in
adipocytes was also observed [106], and GH secretion in fasted rats was stimulated by leptin administration [112]. Leptin is also a regulator of kisspeptin neurons in the ARC [113]. Kisspeptin is involved in the regulation of pulsatile gonadotropin-releasing hormone (GnRH) secretion, which sequentially stimulates the pituitary release of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) to initiate puberty or regulate ovulation through the promotion of sex steroid hormone (i.e. testosterone in men and estrogens in women) secretion [114]. Therefore, lowered leptin contributes to the reduced kisspeptin levels and GnRH secretion, leading to lower testosterone and estrogen secretion in fasting males and AN females, respectively [87, 98, 107, 111]. Testosterone upregulates the expression of β-adrenergic receptors, which further activates protein kinase A to phosphorylate enzymes involved in lipolysis [70]. Meanwhile, lipid uptake is prevented by testosterone via downregulating lipoprotein lipase (LPL) activity [115]. Testosterone administration reduced vWAT accumulation in men [116] as well as the adipocyte size in hypogonadal male rats [117]. Therefore, decreased secretion of testosterone may be associated with the observed maintenance of percentage trunk fat while reducing extremity fat in adolescent boys with AN [89]. Estrogen was also inversely associated with fat attenuation in both sWAT and vWAT in AN females [98]. Chronically reduced secretion of estrogen causes amenorrhea in female AN patients, and resumption of menses is considered to be a biological indicator of health status after weight restoration, which is associated with a higher percentage of body fat mass [118]. Interestingly, plasma kisspeptin is positively correlated with BMI and body fat mass in premenopausal adult females with AN [119] while inversely correlated with BMI in adolescent girls [120], indicating different regulatory patterns of hypothalamic–pituitary–gonadal axis activation in adolescent and adult females in the AN state. Although mRNA expression of leptin in abdominal sWAT was
decreased in AN [121], free leptin concentrations in abdominal sWAT were similar to those in the healthy state [97]. In contrast, increased mRNA expression of resistin, an adipose-derived hormone that is highly expressed in the abdomen and is involved in insulin secretion and energy balance, was associated with greater amounts of the hormone in abdominal sWAT [110], despite that circulating resistin was either unchanged [121] or decreased [110] in AN patients compared to the healthy counterparts.

Chronic starvation activates the stress cascade in the hypothalamus to further interfere with the hormonal regulation of adipose tissue physiology. One critical factor responsible for stress cascade activation is corticotropin-releasing factor (CRF), also referred to as corticotropin-releasing hormone. Upon starvation, increased CRF expression in the paraventricular nucleus (PVN) promotes the release of adrenocorticotropic hormone (ACTH) from the anterior pituitary [122]. ACTH then targets the adrenal cortex to stimulate the production of cortisol, which facilitates lipid accumulation in the presence of insulin while promoting lipid mobilization in the presence of GH [123]. In adult female AN patients, urinary free cortisol was positively correlated with thigh fat while its correlation with cross sectional area in abdominal sWAT and vWAT was negative [98]. Release of CRF onto the ARC also suppresses appetite and promotes energy expenditure by inhibiting NPY/AgRP neuronal activity [124]. Ghrelin can activate CRF neurons in the PVN independent of the mediation of ARC AgRP/NPY neurons, which provides another mechanism for elevating cortisol levels in the fasting state [125]. Elevated cortisol also suppresses the release of thyroid-stimulating hormone (TSH) from the anterior pituitary, which is also decreased by ghrelin [126]. TSH sequentially activates triiodothyronine (T3) [127]. Thus, lowered T3 was observed together with low BMI and leptin levels as well as increased ghrelin and cortisol, in the AN state [87, 107, 108]. T3 plays an important role in regulating energy
balance. Reduced circulating T3 results in decreased resting energy expenditure and lipolytic activities [128], which is pivotal in the conservation of energy for vital functions under the AN or fasting state. Cortisol, ghrelin [129] and GH [130] are positively correlated, while leptin [131], IGF-1 [129], testosterone and estrogen [132] as well as T3 [133] are negatively correlated with MAT mass. Hence, increased cortisol, ghrelin and GH, accompanied by reduced leptin, IGF-1, sex steroid hormones and T3 in AN, are also associated with suboptimal bone health.

There is limited knowledge on the regulation of lipolysis in AN. Although lipolysis can contribute to the energy requirement for maintenance under a negative energy balance state, in adult female AN patients, fasting plasma glycerol [134, 135] or free fatty acids (FFA) [136] did not differ from age-matched healthy controls, whereas glycerol concentrations in sWAT were indeed greater in the AN state [134]. In adolescent girls with AN, fasting plasma glycerol as well as glycerol production varied considerably among patients. Although glycerol production rate was inversely correlated with body weight, suggesting that there is more lipolytic activity in those with lower body weight, no such correlation was observed between glycerol and body weight or BMI [137]. Since the duration or severity of AN in each patient was not specified, it is not known whether such differences contributed to the variation among patients. It is possible that during the early stage of AN, lipolysis increases to facilitate a return to homeostasis, whereas under prolonged fasting-induced negative energy balance, lipolysis decreases to preserve energy required for vital maintenance. This postulation is supported by the observations that adult female AN patients had lower heart rate and blood pressure than their healthy counterparts [134, 135]. Moreover, a recent study also revealed that NPY-knockout mice displayed greater rates of lipolysis, fat mass loss and mortality during prolonged calorie
restriction than wild-type healthy controls [138], suggesting that altered gene expression among patients may also contribute to the variations in lipolysis.

2.4 Genetic association of AN and lipid metabolism

Based on family and twin studies, the symptoms/behaviors of AN are heritable, where genetic heritability explains about 46-72% of the eating behavior (restrictive and binge-purging/vomiting) and 32-72% of the pathological attitudes (e.g. body dissatisfaction, weight concern and weight preoccupation), with the higher heritability observed in post- than pre-pubertal twins [139]. Moreover, familial co-transmission of psychological (major depression and anxiety) and personality traits (reactivity towards stress, negative emotionality and harm avoidance) both contributed to the pathogenesis of AN [139]. The estimated overall heritability of AN was 0.56 based on the large cohort twin study [140]. The partitioning single-nucleotide polymorphism (SNP) heritability of AN was 0.20, indicating the contribution of genetic variation on twin-based heritability of AN [141]. As reviewed in [142] and [143], SNPs in the genes encoding serotonin receptors, serotonin transporter, dopamine receptors DRD2 and DRD4, catechol-O-methyltransferase (COMT), leptin and leptin receptor, ghrelin, melanocortin 4 receptor (MC4R), proopiomelanocortin (POMC), AgRP, estrogen receptors ESR1 and ESR2/ESβ, brain-derived neurotrophic factor (BDNF), cannabinoid receptor 1 (CNR1), opioid receptor delta 1 (OPRD1), as well as fat mass and obesity associated gene (FTO) are associated with the risk of AN. Among these genes, serotonin receptors, serotonin transporter, dopamine receptors, COMT, ghrelin, MC4R, POMC, AgRP, BDNF, CNR1 and OPRD1 indirectly affect adipose tissue metabolism through appetite regulation/body weight regulation in the central nervous system, whereas leptin and leptin receptor, estrogen receptors and FTO directly act on adipose tissue to regulate lipid metabolism.
A recent genome-wide association study (GWAS) revealed one locus with genome-wide significance for a single variant on chromosome 12, which covers 6 genes including zinc finger CCCH-type containing 10 (ZC3H10) and receptor tyrosine-protein kinase ERBB3 [141]. ZC3H10 is an activator of UCP1 and promotes brown adipocyte differentiation, and transgenic overexpression of Zc3h10 prohibits diet-induced obesity [144], while the expression of ERBB3 was downregulated during lipogenesis in sebocytes [145]. Additionally, there was a positive genetic correlation between AN and high density lipoprotein (HDL) cholesterol, and negative correlation between AN and BMI, insulin, glucose, low density lipoprotein cholesterol (LDL) and very low density lipoprotein cholesterol [141].

Gene-tissue associations from a transcriptomic imputation study attributed 13.9% of the phenotype variance in AN to sWAT [146]. Moreover, decreased expression of receptor accessory protein 5 in the dorsolateral prefrontal cortex, which promotes the expression of olfactory receptors and is positively correlated with body weight, is associated with a higher risk of AN [146], probably due to the dysfunction of odor discrimination and sequential desire of food.

Transcriptomic analysis on cortical neurons differentiated from induced pluripotent stem cells derived from AN and healthy females revealed higher expression of the TSH receptor, which potentiates adipogenesis in vitro while inhibiting fatty acid synthase expression for lipogenesis [147], under the AN state [148]. Meanwhile, the expression of diacylglycerol kinase gamma, which has increased expression in the prefrontal cortex under chronic stress exposure [149] and decreased expression in sWAT and vWAT under the obese state [150] in mice, was also increased under the AN state, indicating that aberrant regulation of the genes involved in lipid metabolism may contribute to the pathogenesis of AN in a synergistic manner [148].
2.5 Epigenetic modifications in AN and the effect on adipose tissue metabolism

Epigenetic modification refers to the heritable alteration of gene expression without changing the DNA sequence, and includes modifications such as methylation, acetylation, phosphorylation, ubiquitylation and SUMOylation that are attached to either the DNA or histone proteins [151]. According to the most recent review on epigenetics and eating disorders, thus far, human studies have only assessed DNA methylation status and exclusively in female patients [152]. Whole blood-based measurements revealed hypomethylation in both adolescent and adult AN females, while buccal cell-based and lymphocyte-based measurements showed unchanged and increased global DNA methylation in adult AN patients, respectively [152]. Despite the association of SNPs in appetite regulatory genes POMC, CNR1, BDNF, SLC6A4 (encoding serotonin receptor), DRD4, and adipose tissue regulatory gene LEP (encoding leptin) with AN, methylation status of these genes did not differ between AN and control subjects [152]. The epigenome-wide association studies (EWAS) revealed differentially methylated genes related to lipid metabolism between AN and healthy control subjects [152, 153], whereas the lack of follow-up gene expression measurement made it difficult to draw convincing conclusions.

Although histone modifications have not been comprehensively studied in AN, histone deacetylase 4 (HDAC4)—an enzyme that facilitates the removal of acetyl groups from histones, is differentially methylated in AN and other eating disorders (reviewed in [154]). Altered methylation status of several 5'-cytosine-phosphate-guanine-3' (CpG) sites in the promoter region of HDAC4 has been associated with serum estrogen, fear, learning, appetite and body weight regulation as well as reward processing, etc., which share similar symptomatology with AN [154]. In thigh fat, hypermethylation of HDAC4 resulted in lowered mRNA expression but higher lipogenic activity after long-term exercise intervention in healthy men [155]. These
results collectively suggest a role of HDAC4 in the pathogenesis of AN regarding both appetite regulation and adipose tissue metabolism. A pilot study also revealed that there was hypermethylation of several CpG sites in exon 1 and the MT2 region of the oxytocin receptor gene in the buccal cells of female AN patients compared to the healthy controls, and the methylation level was inversely associated with BMI [156]. Circulating oxytocin is lower in AN patients than healthy counterparts, which is related to the reduced estrogen level—the modulator of oxytocin secretion in AN as well as its blunted response towards estrogen stimulation under the AN state [157]. As reviewed in [157], oxytocin promotes adipogenesis, lipogenesis, as well as lipolysis both in vitro and in the WAT of healthy lean rodents in vivo, and it also suppresses reward-driven but not hunger-driven food intake, indicating its essential role in maintaining energy homeostasis. Therefore, hypermethylation of the oxytocin receptor gene may induce unbalanced energy homeostasis together with other factors such as estrogen, which eventually contribute to the pathogenesis of AN.

3. Stress-induced epigenetic changes in energy metabolism and pathogenesis of AN

The environmental factors include but are not limited to stress exposure (e.g. psychological, physiological, nutritional, etc.), chemical exposure (e.g. pollutants and drugs/medications), and seasonal/biological rhythm, etc. Epigenetic modification is a major mechanism through which such factors contribute to the pathogenesis of AN. Stress-induced epigenetic changes in energy metabolism have been studied extensively [158-160]. Female singletons whose mothers were exposed to the Dutch famine during gestation had higher circulating total cholesterol, triglycerides and LDL cholesterol than unexposed offspring [159]. A later study reported the association of famine exposure and LEP hypermethylation in only the male offspring regardless of the gestational stage of exposure [161]. Similarly, recent research on the offspring of mothers
who suffered from the Chinese famine during pregnancy revealed increased total cholesterol levels [162] and LDL cholesterol levels [163] in their adulthood. Moreover, such exposure only increased the risk of dyslipidemia in females, but not males, during adulthood [163], suggesting a sex difference to the susceptibility of abnormal lipid metabolism upon early-life nutritional stress exposure.

As mentioned in 2.3, upon stress exposure, the hypothalamic-pituitary-adrenal (HPA) axis is activated by CRF. In adult mice, long-term social defeat stress exposure, which induces anhedonia, led to decreased DNA methylation in the promoter region of the Crf gene and consequently higher mRNA expression in the PVN [164]. Greater gestational CRF concentration is associated with increased risk of fetal growth restriction [165], where the fetuses are smaller in abdominal circumference and lower in abdominal, mid-arm and mid-thigh fat masses compared to the ones that developed without gestational CRF fluctuation [166]. Meanwhile, there is greater adipogenic and lipogenic but lower lipolytic activity in the adipose tissue of those growth-restricted fetuses, accompanied with increased risk of visceral adiposity later in life [167]. In contrast, exposure to 14-day chronic variable mild stress only increased methylation of the overall CpG sites in the promoter region of exon 1 and of the intronic sequence between exons 1 and 2 of the Crf gene in female adult rats, accompanied with decreased number of CRF-immunoreactive neurons in the PVN, whereas only in males there was increased Crf mRNA-positive cells in the PVN, although both sexes had lower body weights than their non-stressed counterparts [168]. Such a sex difference in stress-induced methylation and the stress response was postulated to be related to the higher basal corticosterone (the form of cortisol in rodents) level in the females [168].
Activation of CRF promotes the secretion of cortisol, which then binds to the glucocorticoid receptor (GR) to provide negative feedback to the HPA axis and suppresses the stress response to restore homeostasis [169]. Maternal stress exposure during pregnancy increased GR methylation in the promoter region [170] as well as in exon 1F [171] of the offspring. This methylation was associated with higher cortisol stimulation under stress in the offspring [172], which may lead to consistently greater circulating cortisol levels during chronic stress exposure—in line with the observations in the AN patients. Elevated cortisol is associated with the accumulation of vWAT [173] and persistent elevation of corticosterone also impaired non-shivering thermogenesis and promoted lipid storage inside the BAT in rats [174]. Indeed, in rats, maternal stress exposure during gestation induced higher baseline corticosterone but lower insulin in the offspring together with defective responses to leptin and ghrelin upon starvation, leading to food intake refractory responses and weight loss [175]. These symptoms are similar those observed in AN patients, indicating that prenatal stress exposure plays a role in the pathogenesis of AN later in life.

4. Adipose tissue physiology under obese state

4.1 Fat mass and adipose tissue distribution

World Health Organization (WHO) defines obesity as “abnormal or excessive fat accumulation that may impair health” and a BMI ≥ 30 is classified as obese in adults, whereas for young children and adolescents, the standard was made according to the WHO Growth Reference [176]. Current studies also adopt a percentage body fat ≥ 25% in men and ≥ 35% in women as a criterion for obesity diagnosis [177]. Gender, age and ethnic group are the major variables predicting fat mass in obesity [178]. US National Health and Nutrition Examination Survey
(NHANES) reported greater prevalence of high adiposity (on the basis of the 80th percentile of percentage body fat) in Mexican-Americans than non-Hispanic (NH) white and black males but not females aged 8-19 years [179]. In adults, fat mass tended to be higher in black men and women compared to white men and women, respectively, at a fixed BMI [180]. Young adults (18-29 years) had a lower percentage of fat mass than the elderly (≥ 70 years), and there were differences among ethnic groups (Mexican-American > NH white > NH black) whereas there was no difference among these groups in the elders regardless of sex [181]. Moreover, without a significant difference in BMI, middle-aged overweight/obese women had higher percentage body fat masses than men [182]. According to NHANES 1999-2004, percentage extremity fat (of the total mass in the corresponding arm/leg) in males from all ethnic groups decreased during adolescence then increased again throughout later life at the same percentile of the age groups, whereas in women, percentage of extremity fat continued increasing until 79 years of age, then dropped slightly in later life. Moreover, in males among all ethnic groups, percentage extremity fat had a lower reduction during adolescence and greater increase in later life in those in the ≤ 50th percentile than those in the > 50th percentile. The reduction in percentage leg fat continued until 59 years of age before it went back up in those in the > 50th percentile. In females among all ethnic groups, percentage extremity fat had a greater increase until 79 years of age in those in the ≤ 50th percentile than those in the > 50th percentile, whereas the decrease later in life did not differ among percentiles. In males among all ethnic groups, like the percentage extremity fat, the percentage trunk fat reduced during adolescence and then increased again throughout later life, with a slight reduction after 79 years of age. This reduction during adolescence was higher in those in the > 50th percentile than those in the ≤ 50th percentile in Mexican Americans, but not NH white or black individuals, while the increase later in life was lower in those in the > 50th
percentile among all ethnic groups. In females among all ethnic groups, there was a greater increase in percentage trunk fat until 79 years of age in those in the ≤ 50th percentile, whereas a greater reduction from 79 years of age on was only observed in the ≤ 50th percentile of Mexican Americans [183]. At BMI = 30, Chinese and South Asians (adjusted for age and sex by cohort means) had more vWAT than the Europeans, while sWAT was similar between Chinese and Europeans but greater in South Asians [184].

Brown fat also changes during the progression to obesity. BAT activity is greater in lean healthy females than males, and in young people than in elders [185]. In BAT-active subjects, BAT function is inversely correlated with BMI [186] as well as percentage body fat mass, whereas those with BMI > 35 and percentage body fat mass > 30% barely showed any detectable BAT activity [187]. Specifically, obese males had a lower percentage of active BAT volume in all 6 depots (cervical, supraclavicular, axillary, mediastinal, paraspinal and abdominal) and lower BAT activity in 5 depots (except cervical) than their lean healthy counterparts. The greatest difference in the volume was observed in the paraspinal depot, while the activity difference differed the most in supraclavicular depot—the most BAT active depot in healthy lean males, between lean and obese males [188].

4.2 Depot-specific adipose tissue physiology

Obese female adults store more dietary fat in sWAT, especially in the lower body, whereas vWAT is the preferred depot for dietary fat storage in obese males [189]. A recent study identified 414 differentially-expressed genes between sWAT and vWAT, among which, 60 were associated with obesity-related traits (e.g. waist to hip ratio)[190]. Pathway analysis revealed that genes involved in extracellular matrix (ECM) remodeling were highly enriched in the sWAT,
which may confer the functional morphology of adipocytes in sWAT to exert a protective effect, while genes involved in inflammation were highly enriched in vWAT, which confirms its role in the pathogenesis of metabolic diseases. Moreover, developmental genes belonging to the homeobox (HOX) family were also differentially expressed in sWAT and vWAT, with HOXC and HOXD clusters being highly expressed in sWAT and HOXB being highly expressed in vWAT, which may contribute to the functional characteristics of the depots [190]. Moreover, genes related to adipocyte metabolism (proliferation, differentiation, lipid turnover, etc.), adipokine secretion, ion channel and cell signaling are also differentially expressed in sWAT and vWAT, according to transcriptome studies [191-193].

Upon exposure to excessive lipids in the circulation, hypertrophy occurs to adapt to extra energy storage, which increases fat mass and triggers hyperplasia [83]. Preadipocytes from sWAT express more of adipogenic master regulators PPARγ and C/EBPα, thus are more capable of extensive replication and rapid adipogenesis than the preadipocytes from vWAT. The vWAT preadipocytes, however, show a catch-up growth pattern after 60 days of culturing in vitro [189]. Additionally, an increase in endocannabinoids, which promote adipogenesis and triglyceride uptake through the upregulation of PPARγ, was only observed in visceral obesity [194]. Postprandial FFA release is greater in abdominal than lower body obesity, indicating a faster rate of lipolysis in the visceral adipocytes which are inherently resistant to the antilipolytic effect of insulin. Moreover, vWAT is more susceptible to TNFα-induced apoptosis, whereas sWAT is more sensitive to aging and sequential loss of lipid storage capacity (reviewed in [189]).

Expansion of adipose tissue under the obese state has been categorized into two types: metabolically healthy—with preferred lipid storage in sWAT, and metabolically unhealthy—with excessive vWAT and ectopic fat accumulation [195]. Accumulation of sWAT in the lower
body is associated with lowered circulating triglycerides and glucose but greater HDL and insulin sensitivity, while accumulation of abdominal sWAT may be involved in the pathogenesis of insulin resistance and cardiovascular diseases, although the association is not as strong as for vWAT [194]. Impaired expandability of sWAT upon excessive lipid storage can result in the accumulation of vWAT [195]. As vWAT attaches and shares the vasculature with internal organs, lipid metabolites and adipokines are directly secreted from these adipocytes into the portal vein, leading to the disruption of homeostasis in hepatocytes and sequential impacts on gluconeogenesis and lipid metabolism, which eventually contributes to the onset of insulin resistance [194]. Apart from sWAT and vWAT, interestingly, in contrast to the increased MAT accompanied with lowered mineral density in AN, obese adolescents [196] and adults (excluding postmenopausal women) [197] had less MAT with greater bone mineral density than healthy controls, while postmenopausal obese women had increased MAT due to the lack of estrogen [198]. However, the association between MAT and total fat/vWAT/sWAT masses were controversial among studies, which may suggest a unique role of MAT in endocrine modulation and the progression of adiposity [197]. To the contrary, both itMAT and iMAT increased in obese subjects, but only itMAT had a similar distribution and association with inflammation and insulin sensitivity as vWAT [6].

Rapid expansion of adipose tissue with low vascularization induces hypoxia in obesity. The hypoxic adipocytes secrete chemokines which attract macrophages and trigger the inflammatory responses [199]. Many proinflammatory cytokines, including but not limited to those from the interleukin cytokine family, interferon γ, TNFα, monocyte chemoattractant protein 1, and plasminogen activator inhibitor 1, etc., are more highly expressed in vWAT than sWAT, which may be related to the enriched lymph nodes and milky spots for immunocompetent molecules in
visceral depots such as the omentum (reviewed in [189, 194]). Hypertrophy of adipocytes from vWAT under the obese state stimulates the secretion of proinflammatory cytokines in the adipose tissue and results in increased vascular permeability, which attracts macrophages. Increased infiltration and proliferation of macrophages in adipose tissue sustains the inflammatory response, and further stimulates lipolysis in the surrounding adipocytes to provide energy for the immune system. The increased circulating FFAs in turn facilitate the maturation of preadipocytes and adipocyte hypertrophy, which ultimately leads to metabolic dysregulation [200, 201].

Interestingly, BAT also recruits macrophages and secretes proinflammatory cytokines, which trigger the inflammatory response. Inflammation in BAT inhibits the proliferation of brown adipocytes through the interruption of catecholamine signaling and the differentiation of brown adipocytes by downregulating PPARγ, while promoting apoptosis of brown adipocytes via the stimulation of TNFα, which together alter the thermogenic activity of BAT (reviewed in [202, 203]).

**4.3 Endocrine disruption in obesity**

Although rapid accumulation of adipose tissue in the obese state is accompanied by elevated leptin, which regulates energy homeostasis by suppressing food intake and promoting energy expenditure, decreased amounts of soluble leptin receptors as well as reduced leptin transport across the blood brain barrier (BBB), and reduced leptin signaling in hypothalamus result in hyperleptinemia and eventually leptin resistance [204]. Leptin suppresses food intake by upregulating *POMC* mRNA expression while inhibiting *NPY/AgRP* mRNA expression in the ARC (reviewed in [205]). Reduced leptin transport across the BBB leads to decreased production
of POMC, which suppresses food intake [206]. Mice with the leptin receptor gene knockout in brain endothelial and epithelial cells consumed more of a high fat, but not chow diet, and had more body fat, indicating a higher sensitivity to food reward under the obese state due to reduced leptin receptor expression [207].

Leptin promotes lipolysis and fatty acid oxidation in adipose tissue as well as insulin-stimulated glucose uptake and oxidation in muscle while inhibiting hepatic gluconeogenesis and insulin synthesis and secretion in the pancreas [208]. Leptin resistance thus results in decreased lipolysis, fatty acid oxidation and insulin sensitivity and increased hepatic glucose production as well as circulating insulin. Increased circulating glucose and insulin together with decreased insulin sensitivity contributes to hyperglycemia and hyperinsulinemia, while excessive circulating lipids resulting from decreased fatty acid oxidation contribute to hyperlipidemia [206]. Detailed molecular mechanisms of leptin regulation of glucose and lipid metabolism are reviewed in [209].

In contrast to the negative energy balance-induced increase in ghrelin under the AN state, under the obese state, ghrelin is reduced due to excessive energy intake [210]. UCP2, which is positively associated with ghrelin has decreased expression in obese subjects, with more of a reduction in the abdominal vWAT than sWAT compared to the lean healthy controls, regardless of an overall greater expression in vWAT than sWAT. Moreover, sWAT UCP2 is positively correlated with both circulating and depot-specific adiponectin levels, and lowered adiponectin is associated with insulin resistance and dyslipidemia in obese subjects [211]. In contrast, expression of UCP2 in pancreatic islets was higher in obese than lean mice, which further led to reduced ATP production and decreased secretion of insulin [212]. Increased β-cell mass was observed in diet- and gene mutation-induced obese mice, which may compensate for the
decreased secretion of insulin and the exposure to increased glucose under the obese state [213]. However, the expansion of β-cell mass was coupled with cellular dysfunction which led to reduced insulin sensitivity and eventually insulin resistance (detailed mechanisms reviewed in [214]).

As the endogenous ligand of the GH secretagogue receptor, reduced ghrelin is associated with lowered GH levels in obesity [215]. Decreased GH release from the anterior pituitary to the circulation and faster GH clearance may contribute to declined synthesis of IGF-1 in adipose tissue (although circulating IGF-1 tends to be normal in most studies), which together are associated with reduced lipolysis and the accumulation of fat, especially in vWAT [216].

Decreased expression of kisspeptin in the ARC was observed in female mice that are centrally resistant to leptin signaling and prone to obesity-induced infertility [217]. Similarly, in HFD-induced male mice, increased circulating leptin and decreased testosterone and LH were observed together with decreased expression of leptin receptor, kisspeptin, and GnRH, indicating a role in obesity-induced male hypogonadism [218]. As mentioned above, leptin regulates kisspeptin neurons in the ARC, which promotes sex hormone secretion via modulating GnRH pulse. Consistent with this, lower LH and FSH levels and lower sex hormone binding globulin (SHBG) were observed in both premenopausal [219] and postmenopausal [220] obese females, and lower total testosterone, FSH and SHBG were observed in obese males compared to their respective healthy counterparts [221]. However, total estrogen was lower in premenopausal but higher in postmenopausal obese females compared to the respective age-matched healthy counterparts [222] with no change in free estrogen regardless of the menopausal state, although the overall estrogen level was higher in premenopausal females [223]. Both free and total testosterone levels increased in obese females regardless of the menopause state compared to the
healthy counterparts, with an overall higher testosterone level in premenopausal females [223]. In contrast, there was more free estrogen in obese than healthy males [221]. In males, lowered hepatic production of SHBG due to the disruption of endocrine homeostasis under the obese state, such as hyperinsulinemia, resulted in lowered binding of testosterone and thus more free testosterone. As adipose tissue expansion promotes the production of aromatase, an enzyme that converts free testosterone to estrogen, low total testosterone is often observed in obese males, which alters the negative feedback from GnRH and further induces hypogonadism [224]. In the healthy state, testosterone stimulates catecholamine-induced lipolysis and inhibits triglyceride uptake in abdominal adipose tissue. Thus, reduced testosterone in obese males contributes to increased vWAT accumulation to further exacerbate obesity, forming a “self-perpetuating cycle” [225].

Intriguingly, in contrast to the lowered intra testicular testosterone in obese males, in obese females, hyperinsulinemia promotes the ovarian production of androgens, leading to increases in both total and free testosterone, or hyperandrogenism [224]. Estrogen production in postmenopausal females is solely dependent on the conversion of androgen by aromatase, whereas premenopausal females can also produce estrogen in functional ovaries and the metabolic clearance rate is higher than that in postmenopausal females [226]. Therefore, increased circulating estrogen in postmenopausal obese females may due to the conversion of androgen while decreased levels in premenopausal obese females may be attributed to simultaneously increased clearance of estrogen [222]. An increased clearance of estrogen in premenopausal obese females may be protective for ovary functions as hyperestrogenism may induce endometrial hyperplasia and thereby impair fertility [224]. Estrogen upregulates the expression of antilipolytic α2A-adrenergic receptor only in sWAT [227]. Meanwhile, the
estrogen receptor is highly expressed in sWAT in females [228]. Thus, premenopausal females preferentially deposit fat in sWAT. Due to the dramatic reduction in endogenous estrogen and increase in androgens, fat accumulation shifted from sWAT to vWAT in postmenopausal females, leading to the increased propensity for metabolically unhealthy obesity [228]. Moreover, estrogen suppresses appetite through the direct stimulation of central POMC/cocaine-amphetamine-regulated transcript (CART) neurons and inhibition of NPY/AgRP neurons in the ARC, as well as potentiating the release of satiating cholecystokinin peptide from the small intestine upon food consumption. Testosterone, in contrast, increases food consumption frequency through the suppression of POMC/CART [229]. Thus, fluctuation of sex hormones under the obese state may exacerbate fat mass accumulation and endocrine disturbances through the promotion of food intake.

Hyperactivation of the HPA axis is observed in obesity. There is greater CRF and ACTH release in obese than lean subjects, whereas increased urinary-free cortisol was only observed in abdominal obesity [216]. This may be explained by the fact that vWAT has more abundant mineralocorticoid receptors (MR), which have higher cortisol binding affinity than GR, compared to sWAT, and that the expression of MR in human fat, especially vWAT, is positively related to BMI [204]. Elevation of cortisol in obesity results from the expansion of adipose tissue mass, where the highly expressed enzyme 11-hydroxysteroid dehydrogenase type 1 converts the inactive cortisone to active cortisol [224]. Cortisol promotes hepatic gluconeogenesis whereby insulin secretion is stimulated, and LPL activity is enhanced in abdominal adipose tissue to further exacerbate vWAT accumulation [216]. Such alterations in the HPA axis in the obese state lead to abnormal responses to environmental stressors, which is discussed in section 2.2.6.
Circulating TSH is positively correlated with BMI and leptin, although many obese subjects had a similar thyroid hormone profile as healthy counterparts. Free T3 and total T3 levels in obese subjects were further positively correlated with TSH [230]. The slightly increase in TSH and T3 may result from enhanced gene expression in the PVN guided by leptin [231]. Increased T3 was postulated to be an adaptation to the positive energy balance under the obese state as T3 promotes energy expenditure [232]. However, lower expression of TSH and T3 receptors were found in the adipose tissue of obese patients, leading to more free TSH and T3 but less negative feedback, which further stimulates the production of TSH and T3, eventually leading to thyroid hormone resistance and thyroid dysfunction [231] (summarized in Figure 2.4).

4.4 Genetic association of obesity and lipid metabolism

There are two types of genetic obesity—monogenic, which results from a single gene mutation with or without syndromes like cognitive defects, and polygenic, which is caused by the interplay of multiple genes with moderate interaction with the environment [233]. Monogenic obesity is rare and often causes early-onset and severe obesity. The genetic causes of syndromic monogenic obesity are not fully elucidated to date, while all genes that contribute to non-syndromic monogenic obesity are involved in energy homeostasis through the leptin-melanocortin pathway (reviewed in [234]). Polygenic obesity is the most common form of obesity. Meta-analysis of large-scale GWAS identified 941 near-independent BMI-associated SNPs (summarized in [234]), most of which were within 138 differentially expressed genes involved in neurogenesis and the development of central nervous system [235]. Loci associated with obesity overlapped with genes involved in appetite and energy regulation, lipid metabolism and adipogenesis, as well as insulin secretion and action [233]. Moreover, both familial and twin studies showed that total body fat mass as well as regional fat mass (e.g. trunk fat and lower
body fat) are heritable [236]. A recent GWAS revealed SNPs in/near 25, 6 and 26 genes known to be independently associated with arm, leg and trunk fat ratios (calculated by dividing corresponding depot fat mass by total body fat mass), respectively, with another 36 genes being associated with both leg and trunk fat ratios, 3 genes associated with both arm and trunk fat ratios and 1 gene associated with both arm and leg fat ratios (summarized in [237]). Only one locus near the gene a disintegrin and metalloproteinase with thrombospondin motifs-like 3, which was previously found to be associated with height and waist and hip circumferences [238], was associated with fat ratios in all three depots [237]. Sex-stratified analyses further revealed higher SNP heritability of all traits in females (~21-25 %) than in males (~11-15 %), with the majority of variants being associated with leg and/or trunk fat ratios [237]. SNPs of genes related to ECM maintenance and remodeling as well as cell-ECM interaction enriched in reproductive, musculoskeletal and adipose tissues were highly associated with leg and trunk fat ratios in females, but not males, indicating a critical role of ECM regulatory genes in the variability of female body fat distribution [237]. One study using genetic risk score, which aggregates multiple SNPs as they are inherited together from the parents, suggested that genes involved in sex hormone as well as SHBG productions were also associated with vWAT accumulation [11]. Additionally, differences in sex hormone production and sex-specific body fat distribution patterns are also associated with the number of X chromosomes, as males with an extra X chromosome (e.g. Klinefelter syndrome) had less testosterone production and higher abdominal adiposity, which may be explained by the variants of genes inducing the escape of X inactivation [11].
4.5 Epigenetic modifications in obesity and effects on adipose tissue metabolism

One of the first large-scale EWAS in adults from European origin revealed that methylation in intron 1 of hypoxia inducible factor 3 alpha subunit gene (HIF3A), which regulates cellular oxygen concentration and promotes adipocyte differentiation, was positively correlated with BMI in both whole blood and sWAT [239]. Further investigation revealed that methylation of the same locus in HIF3A was higher in vWAT than sWAT, which corresponded with lower mRNA expression in vWAT than sWAT in the middle-aged Caucasian population. HIF3A methylation in vWAT was positively correlated with sWAT, vWAT, and hip fat masses and negatively correlated with adiponectin levels after adjusting for age, gender and BMI, while that in sWAT was negatively correlated with age [240]. Moreover, greater methylation of HIF3A at the same position in blood DNA was also observed in obese children [241], whereas modulation of HIF3A methylation status by nutrients suggested that methylation is the consequence, but not cause of obesity [242]. Consistent with this conclusion, although EWAS identified over 370 CpG sites being associated with BMI, so far, Mendelian randomization and longitudinal studies provided evidence that changes in BMI preceded the changes in DNA methylation, and the strength of BMI-methylation association varied among ethnic groups [243]. As reviewed in [233], EWAS also showed that differentially methylated genes involved in adipogenesis, lipogenesis, fatty acid oxidation, lipoprotein metabolism as well as insulin signaling and inflammation in sWAT and/or vWAT were related to obesity traits, and about 30% of the methylated loci were further associated with the nearby SNPs, which may suggest a role of SNP-directed DNA methylation and sequential gene expression changes in obesity. A recent methylome-wide association study also demonstrated that males and females with abdominal obesity shared over 75% similarities in methylation patterns in white blood cell DNA [244].
Candidate gene methylation analyses are support the results from EWAS in that methylation in genes involved in appetite regulation and lipid metabolism in adipose tissue was associated with body fat mass and fat distribution, plasma triglyceride and lipoprotein levels, and genes involved in circadian rhythm regulation were also associated with obesity-related traits (reviewed in [233]). It should be noted that most of the EWAS recruited middle-aged Caucasian males and females although epigenetic status changes upon aging and epigenetic patterns and the strength of the association with obesity-related traits differs among ethnic groups [243]. As aforementioned, epigenetic status is also highly influenced by environmental factors such as diet, physical activities and medication, and methylation of the same gene in different tissues or depots may also differ [233]. Therefore, future research should focus on a wider range of age groups in different ethnic groups with the comparison of the methylation status in various tissues/adipose depots.

PPARγ is the master transcriptional regulator that is indispensable for adipogenesis. Decreased adipose tissue expression of PPARγ in obesity is associated with inflammation, lipodystrophy as well as insulin resistance [245]. Methylation of PPARγ in sWAT is positively correlated with vWAT mass [246], and PPARγ expression is lower in vWAT than sWAT [247]. Pax transactivation domain-interacting protein promotes the trimethylation of histone 3 lysine 4 (H3K4) and the enrichment of H3K4 methyltransferases in the promoter region to activate PPARγ [248]. Meanwhile, acetylation of H3K9 and H3K27 in the promoter region of PPARγ increases during adipogenesis, together with downregulation of deacetylases, which inhibits adipogenesis [249]. Activated PPARγ upregulates the expression of SET domain bifurcated 8—a histone lysine methyl transferase that monomethylates H4K20, which in turn further enhances the expression of PPARγ, as well as its downstream targets in adipogenesis [249].
Trimethylated H3K4 was also enriched in the promoter region of \textit{LPL} and \textit{IL-6} in normoglycemic morbid obese subjects (BMI > 40 and glucose < 100 mg/dL), and positively correlated with BMI and insulin resistance [250]. Also, there was reduced histone deacetylase 6 in the abdominal vWAT of obese subjects, which was associated with impaired deacetylation of cell death-inducing DNA fragmentation factor alpha-like effector C, leading to the fusion of lipid droplets and thus the accumulation of fat under the obese state [251].

5. Stress-induced epigenetic changes in energy metabolism and pathogenesis of obesity

As described in section 3, maternal undernutrition during gestation causes hypermethylation of \textit{LEP} and increased risk of dyslipidemia in the offspring. Similarly, maternal overnutrition also induces epigenetic changes in the offspring. Offspring from rats fed a HFD throughout gestation and lactation had heavier body weights, increased sWAT and vWAT masses, and hyperleptinemia at weaning compared to those from low fat diet (LFD) fed dams, and some changes persisted to a later age [252]. Moreover, mRNA expression of \textit{Npy} and \textit{Agrp} decreased, while leptin receptor expression increased in the ARC of the offspring from HFD-fed dams, which was accompanied by hypermethylation of the promoter and enhancer regions of hypothalamic \textit{Pomc} at weaning. While the expression of \textit{Npy} and \textit{Agrp} became similar in LFD-fed offspring from either HFD- or LFD-fed dams, greater leptin receptor expression and hypermethylation in the promoter, but not enhancer of \textit{Pomc} persisted in LFD-fed offspring from HFD-fed dams at 17 weeks after weaning. No difference in methylation was detected in offspring fed either LFD or HFD from LFD-fed dams, indicating the critical role of long-term maternal overnutrition on epigenetic alterations in appetite regulation and adipose tissue physiology in the offspring [252]. Indeed, in term placenta from obese women there was lower expression of leptin receptor and adiponectin receptors, DNA hypermethylation of \textit{LEP} on the
fetal side, as well as hypo- and hypermethylation of adiponectin and adiponectin receptor 2, respectively, on the maternal side, compared to the age-matched healthy women [253]. Changes in the enrichment of dimethylated H3K9—a transcriptional repressor in the promoter of adiponectin and monomethylated H4K20—a transcriptional activator in the promoter of leptin also contributed to maternal obesity-induced alterations in leptin and adiponectin expression in the offspring, which was associated with abnormalities in adipose tissue metabolism [254].

Maternal mental stress is also involved in the pathogenesis of obesity in the offspring through epigenetic modifications. Hypermethylation of glucocorticoid receptor gene NR3C1 and 11β-hydroxysteroid dehydrogenase type 2 (11β-HSD-2) was observed in the cord blood of infants exposed to maternal mental stress [255]. 11β-HSD-2 inhibits the conversion of cortisone to its active form cortisol and is highly expressed in the placenta [256]. Hypermethylation of 11β-HSD-2 and NR3C1 could result in lowered 11β-HSD-2 activity and lowered binding of cortisol, leading to elevated free cortisol levels in the offspring which causes alteration in the HPA axis as well as the propensity to accumulate vWAT [194]. The positive correlation of prenatal stress exposure and offspring glucocorticoids is conserved in 14 vertebrate species according to a meta-analysis [257]. However, there are conflicting results for the effect of maternal stress exposure on offspring cortisol in human studies because cortisol/glucocorticoid levels can be affected by sample source (hair, saliva or urine), sampling time (day or night) and the age of the offspring (at birth, childhood, adolescence or adulthood), etc. [258]. Thus, one should exercise caution in drawing too many conclusions from these studies.

Long-term stress-induced chronic elevation of glucocorticoids results in over-production of leptin from adipose tissue and insulin from the pancreas, thereby reducing the sensitivity of leptin and insulin in the brain, which sequentially leads to leptin and insulin resistance and loss
of appetite suppression by inhibiting NPY/AgRP neurons in the ARC [124]. Meanwhile, chronic stress also enhances the preference for sweet and fatty foods, which is attributed to the alteration of the mesolimbic dopamine reward system due to consistently elevated glucocorticoids [173]. Frequent ingestion of highly palatable foods repeatedly stimulates the reward system and in turn reduces the dopaminergic pathways in the brain, leading to a higher threshold of comfort feelings from palatable food, which results in overeating and obesity [173]. The gene promoters for tyrosine hydroxylase and the dopamine transporter, which are involved in dopamine synthesis and transport, respectively, were highly methylated in the ventral tegmental area (VTA)—the central reward circuitry while those in the hypothalamus were hypomethylated compared to rats without HFD-induced obesity. These methylation differences were negatively correlated with mRNA expression in the brain and corresponded with increased food intake but a blunted response to highly palatable food [259]. Moreover, chronic HFD ingestion also decreased the expression of µ-opioid receptor in the VTA of adult male mice, which was associated with increased DNA and H3K9 methylation, increased binding to methyl CpG-binding protein 2 and decreased acetylation of H3 in the promoter region [260]. Additionally, HFD also induced hypermethylation at the promoter region of Lep and Ppars2 (encoding PPARγ isoform 2 in mice) in the vWAT, but not sWAT, in adult male mice, whereas vWAT mRNA expression and circulating leptin were increased and Ppars2 expression was decreased in vWAT [261]. Increased global methylation in T cells, B cells and T-cytotoxic cells were also observed in the peripheral blood of HFD-fed pigs, and was associated with the increased infiltration of macrophages in vWAT and the maintenance of inflammation in adipose tissue [262]. In summary, these results suggest that abnormalities in appetite regulation induced by chronic stress exposure during adulthood promotes the ingestion of highly palatable food and increases
adiposity through epigenetic modifications of genes encoding factors that are involved in lipid metabolism and the immune response, which together contribute to the onset of metabolic diseases.

6. Current knowledge gap in stress and adipose tissue physiology under anorexic and obese states

When considering the effects of stress-induced epigenetic modifications on energy homeostasis, it should be noted that epigenetic modifications are very sensitive to the type and intensity of the stressors, and unlike experimental animals in which there is strict control of housing and diet, the human epigenome may also be affected by diverse factors such as lifestyle [162]. Meanwhile, epigenetic modifications may not necessarily cause changes in gene expression. Thus, human studies with the inclusion of larger sample sizes, and meta-analyses integrating both epigenetic changes and gene/protein expression are expected for future research and the results should be corrected for environmental variables and interpreted with caution. Moreover, although the effect of stress on epigenetic modification of the HPA axis has been comprehensively studied in humans, little is known about the direct effect of stress exposure, especially during early life, on adipose tissue physiology and sequential effects on the endocrine system, in part due to the ethical limitations. Although such studies have been carried out with rodents, focusing on maternal overnutrition and distress, whether the effects are conserved across species both in mammals and non-mammals is important for the understanding of adaptation mechanisms from an evolutionary perspective. Although it is known that early-life stress exposure causes abnormal activities in the HPA axis in later life, whether such activities differ in those genetically predisposed to be anorexic and obese is not clear. Therefore, animal models including both mammals and non-mammals should be developed and adopted to explore the effects of early-life
stress exposure on both early and later life adipose tissue physiology to further elucidate the mechanisms involved, which will shed light on novel therapeutic targets in stress-induced metabolic diseases.

7. Body weight selected lines of chickens as a model to study stress and adipose tissue physiology under anorexic and obese states

7.1 Introduction of the chicken lines

After consecutive selection for over 60 generations, two lines of chickens, whose foundation stock incorporated seven inbred lines of White Plymouth Rocks [263], differed by more than 10-fold in body weight at eight weeks (56 days) of age [264]. The detailed selection procedure can be found in [263-266]. Chickens from the high body weight-selected (HWS) line are hyperphagic and easily accumulate fat, whereas those from the low body weight-selected (LWS) line have low appetite, some are even anorexic, and all very lean [267]. Other correlated responses to this divergent selection for body weight include embryonic development [268-272], reproductive traits [273-277], immunological characteristics [278-281], as well as metabolism and growth [282-294].

7.2 Metabolic differences between the two chicken lines

From the 5th generation on, the divergent selection for body weight at 56 days of age led to pronounced differences in food intake between HWS and LWS, with increased magnitude in the subsequent generations [295]. Further research revealed that the difference in food intake was a result of increased meal number instead of meal size consumed by HWS chicks compared to
LWS [296]. Over generations, HWS chicks became hyperphagic, while LWS chicks were hypophagic with varying degrees of anorexia [290].

Research has been conducted to elucidate the physiological mechanisms by which appetite was regulated. In 1983, Burkhart et al. showed that LWS hens with ventromedial hypothalamus lesions exhibited symptoms of obesity syndrome, while neither body weight nor feed intake changed in the lesioned HWS counterparts [297]. Comprehensive studies on the hypothalamic regulation of appetite in HWS and LWS chickens have been conducted in recent decades. LWS chicks show higher sensitivities in their food intake response to i.c.v. injection of α-melanocyte-stimulating hormone [298], CRF [299], amylin [300], neuropeptide AF [301], insulin [302], and ghrelin [303], while HWS chicks had lower thresholds for neuropeptide S and calcitonin and calcitonin gene-related peptide [304]. Central injection of galanin [305] and gonadotropin-inhibitory hormone [306] increased food intake in both lines with similar magnitudes. Central administration of human recombinant leptin only reduced food intake in LWS but had no effects on HWS [307], while NPY [308] and obestatin [303] increased food intake in HWS but not LWS. Moreover, after stress exposure at hatch, which was a combination of cold and food/water deprivation, only HWS had increased food intake by i.c.v. NPY administration on day 5 post-hatch, while LWS had unchanged appetite [309]. However, only stressed LWS had increased mRNA abundance of NPY in ARC and CRF in PVN, while gene expression was not altered in HWS by the stress exposure [310]. These results collectively suggest that long-term consecutive selection for body weight altered the hypothalamic satiety mechanisms in HWS and LWS chickens.

Feed utilization efficiency was also altered during the course of selection. Lepore et al. [283] showed that the utilization of certain nutrients was more efficient in HWS compared to LWS.
embryos. Later studies demonstrated that when feeding ad libitum, although HWS gained more weight through greater feed consumption, the feed utilization efficiency was not different between the lines, whereas when feed was restricted to the same amount for both lines, the utilization efficiency was higher in HWS compared to LWS chickens during the first four weeks post-hatch [295]. Along with feed intake and utilization efficiency, HWS chickens also showed faster feed passage rate than LWS counterparts, which indicates a faster feed clearance rate, a more efficient feed utilization, and a sequential rapid growth [311]. Consequently, HWS chickens are able to achieve higher body weights than LWS at the same age.

It was reported periodically during selection that the percentage of body fat was higher in HWS than LWS chickens [312-314]. Further studies revealed that fatty acid biosynthesis, which was measured through liver, adipose, and bone tissue enzymatic activities of nicotinamide adenine dinucleotide phosphate (NADP)-malate dehydrogenase, ATP-citrate lyase, and acetyl-CoA carboxylase, which are involved in fatty acid biosynthesis, were greater in one-year old adult LWS than HWS chickens, and lipolysis, which was evaluated by measuring plasma NEFA concentrations, was also greater in LWS than HWS chickens [315]. In 28-day old chicks under the same feeding regimen, the differences in fatty acid biosynthesis and lipolysis between the lines were consistent with those observed in adult chickens [316]. During the fasted state, chickens from both lines showed decreased enzymatic activities of malic enzyme, citrate cleavage enzyme, and acetyl-CoA carboxylase in liver and bone, while LWS had higher circulating NEFAs and greater NEFA mobilization from adipose tissue compared to HWS [316]. Recently, Zhang et al. found that fatty acid oxidation rate and efficiency in abdominal adipose tissue and metabolic flexibility in skeletal muscle were lower in 56-day old HWS than LWS
These results suggest that the higher percentage of body fat deposition in HWS is related more to decreased lipolysis than the contribution of increased fatty acid synthesis.

The first cellular-level research on adipose tissue in HWS and LWS was conducted by Robey et al. [314], who found that after 18 days of force-feeding, HWS chickens at 280 days of age showed both hypertrophy and hyperplasic responses, which was reflected by fewer unfilled adipocytes. In contrast, a hypertrophic response, namely a greater number of unfilled adipocytes, dominated in their LWS counterparts. Consistent with this result, research on 65-day old line chickens under an ad libitum feeding regimen showed that HWS had larger and fewer abdominal adipocytes than LWS [318].

Further molecular studies revealed that in 5-day old line chicks, intraperitoneal (i.p.) insulin administration led to a more pronounced decrease in blood glucose in HWS than LWS [319]. There was greater hypothalamic NPY, NPYR2 and NPYR5 mRNA in LWS than HWS, and POMC mRNA was greater in HWS than LWS [319]. In 90-day old line chickens, i.p. insulin administration produced a similar effect as it did in the younger chicks [320]. Interestingly, the mRNA abundance of NPY, NPYRI and NPYR5 were greater in HWS than LWS in abdominal adipose tissue [320].

More recently, i.p. administration of NPY to 5-day old line chicks was associated with changes in subcutaneous adipose tissue that were reflective of increased fat deposition (increased glycerol-3-phosphate dehydrogenase activity and up-regulation of adipogenic transcription factors during the first few hours post-injection), and reduced lipolysis (lower circulating NEFAs and down-regulation of adipose triglyceride lipase; ATGL), with line differences indicative of greater rates of lipolysis in LWS and adipogenesis in HWS. For instance, there was greater
expressions of ATGL, perilipin 1, and NPYR1 in LWS than HWS, and greater expression of SCD1, glycerol-3-phosphate acyltransferase 3 and LPL in HWS than LWS [321]. These in vivo studies are consistent with the results obtained from the primary cell culture model [322, 323]. These studies show that NPY and its receptors are involved in central and peripheral energy homeostasis regulation in both lines, but possibly through different mechanisms.

Collectively, the dramatic difference in body weight between the two chicken lines is related to hypothalamic appetite regulation and adipose tissue metabolism, which is similar to that in anorexic and obese human subjects. And alteration of appetite and hypothalamic gene expression in stressed LWS at hatch makes the line a good model to study the pathogenesis of AN induced by early-life stress exposure.
References


Figure 2.1. Regulation of preadipocyte proliferation and differentiation in mammals. Committed preadipocytes express delta-like homolog 1 (DLK1), which inhibits differentiation through its activation of the mitogen-activated protein kinase (MAPK) pathway. Meanwhile, factors such as GATA binding proteins 2 (GATA2) and 3 (GATA3), Krüppel-like factor 2 (KLF2) and KLF7 are also highly expressed in preadipocytes and inhibit differentiation through their interactions with peroxisome proliferator-activated receptor gamma (PPARγ) and CCAAT-enhancer-binding protein α (C/EBPα). Upon hormonal induction, elevated cyclic adenosine monophosphate (cAMP) concentrations subsequently stimulates the expression of C/EBPβ and C/EBPδ, which induce the activation of KLF5 and sterol regulatory element-binding protein 1 (SREBP1). The sequential expression of PPARγ and C/EBPα upregulate a cascade of downstream factors, such as fatty acid binding protein 4 (FABP4) to facilitate the maturation of adipocytes.
Figure 2.2. Overview of lipogenesis and lipolysis regulation in mammals. Hepatocytes take up either circulating non-esterified fatty acids (NEFA) or chylomicrons to synthesize very low density lipoproteins (VLDL), or uses non-lipid source for de novo lipogenesis (mainly in birds and mammals) and subsequently synthesizes VLDL. Lipoprotein lipase (LPL) hydrolyzes VLDL into NEFA, which are taken up by adipose tissue (and cells from other tissues in the body) through fatty acid transport protein (FATP) and fatty acid translocase (FAT). NEFA in adipose tissue are further sequestered for incorporation into triacylglycerol (TAG) by glycerol-3-phosphate acyltransferase (GPAT), acyl-CoA acylglycerol-3-phosphate acyltransferases (AGPAT) and diacylglycerol acyltransferase (DGAT). TAG are stored in the form of a lipid droplet in the mature adipocyte. Increased intracellular cAMP activates protein kinase A (PKA), which phosphorylates both perilipin (PLIN) and hormone-sensitive lipase (HSL), and initiates lipolysis. Comparative gene identification-58 (CGI-58) co-activates adipose triglyceride lipase (ATGL) to hydrolyze TAG, which are further hydrolyzed to NEFA by HSL and monoglyceride lipase (MGL). NEFA are sequentially released into the circulation and taken up by cells in the
liver or can be rapidly re-esterified into TAG in the adipocyte (remodeling). GLUT2: glucose transporter 2; ACACA: acetyl-CoA carboxylase alpha; FASN: fatty acid synthase; ELOVL6: elongation of very long chain fatty acid elongase protein 6; SCD1: stearoyl-CoA desaturase; APOB100: apolipoprotein B 100.
Figure 2.3. Endocrine abnormalities in the anorexic state (AN). Decreased food intake in anorexia stimulates the secretin of ghrelin in the stomach, which promotes food intake by upregulating agouti-related peptide/neuropeptide Y (AgRP/NPY) neurons in the arcuate nucleus (ARC) of the hypothalamus. Ghrelin acts on the somatotrophs in the pituitary to stimulate the release of growth hormone (GH) to maintain balance in lipid deposition and mobilization, meanwhile activating corticotropin-releasing factor (CRF) neurons in the paraventricular nucleus, which counteracts effects on AgRP/NPY and inhibits food intake. Under AN, increased GH secretion is accompanied by lowered GH receptor expression and binding capacity in the liver, leading to GH resistance and further reduced effects on inhibiting lipid mobilization and
stimulating insulin-like factor 1 (IGF-1) expression. In turn, IGF-1 provides weak negative feedback to GH production, which further promotes GH resistance. Ghrelin also inhibits insulin secretion by modulating ATP production so as to maintain euglycemia. Chronic fasting under is also a stressor that directly upregulates the expression of CRF to activate the hypothalamic-pituitary-adrenal (HPA) axis. Elevated cortisol sequentially suppresses the release of thyroid-stimulating hormone (TSH), which then lowers the activation of triiodothyronine (T3) via the hypothalamic-pituitary-thyroid (HPT) axis to reduce energy expenditure. Loss of fat mass under AN also reduces the production of leptin, which further downregulates the expression of kisspeptin in the ARC. Reduced kisspeptin then leads to reduced secretion of sex hormones through the regulation of the hypothalamic-pituitary-gonadal (HPG) axis, which eventually weakens lipid mobilization. Therefore, under AN, prolonged negative energy balance may result in reduced lipid mobilization to preserve energy for vital maintenance.
Figure 2.4. Endocrine disruptions under the obese state. Increased fat mass in obesity results in greater secretion of leptin from adipose tissue, while the expression of leptin receptor and transport of leptin across the blood-brain barrier (BBB) are reduced, leading to lowered leptin signaling and eventually leptin resistance. Sequentially, kisspeptin, which is modulated by leptin, is lowered, leading to hypogonadism in obese males and hyperandrogenism in obese females through the regulation of the hypothalamic-pituitary-gonadal (HPG) axis. Meanwhile, expansion of β-cell mass under the obese state is associated with increased production of insulin, which is further exacerbated by leptin resistance, leading to hyperinsulinemia. Hyperinsulinemia also contributes to the disturbance in sex hormones in obese males and females, which eventually
promotes food intake while inhibiting lipid mobilization. Increased food intake inhibits the secretion of ghrelin, resulting in decreased production of growth hormone (GH) and then reduces lipid mobilization. Moreover, an increase in fat mass under the obese state promotes the release of thyroid-stimulating hormone (TSH), which then increases the activation of triiodothyronine (T3) via the hypothalamic-pituitary-thyroid (HPT) axis, while the expression of TSH receptor is lowered under the obese state, leading to reduced negative feedback and thus more release of free TSH and T3 in the circulation. These together, lead to thyroid dysfunction and reduced energy expenditure which eventually results in fat mass accretion. Finally, obesity is associated with increased production of cortisol and hyperactivation of the hypothalamic-pituitary-adrenal (HPA) axis, which abnormally modulates stress responses later on, through which food intake and sequential fat mass are affected.
Chapter 3: Changes in adipose tissue physiology during the first two weeks post-hatch in chicks from lines selected for low or high body weight


Abstract

Chickens from lines selected for low (LWS) or high (HWS) body weight (BW) differ in appetite and adiposity. Mechanisms associated with the predisposition to becoming obese are unclear. The objective of the experiment was to evaluate developmental changes in depot-specific adipose tissue during the first two weeks post-hatch. Subcutaneous (SQ), clavicular (CL) and abdominal (AB) depots were collected at hatch (DOH) and days 4 (D4) and 14 (D14) post-hatch for histological and mRNA measurements. LWS chicks had decreased SQ fat mass on a BW basis with reduced adipocyte size from DOH to D4 and increased BW and fat mass with unchanged adipocyte size from D4 to D14. HWS chicks increased in BW from DOH to D14, and increased in fat mass in all three depots with enlarged adipocytes in the AB depot from D4 to D14. Meanwhile, CCAAT/enhancer-binding protein alpha, neuropeptide Y, peroxisome proliferator-activated receptor gamma and acyl-CoA dehydrogenase mRNAs differed among depots between lines at different ages. Plasma non-esterified fatty acids were greater in LWS than HWS at D4 and D14. From DOH to D4, LWS chicks mobilized SQ fat and replenished the reservoir through hyperplasia, whereas HWS chicks were dependent on hyperplasia and hypertrophy to maintain adipocyte size and depot mass. From D4 to D14, adipose tissue catabolism and adipogenesis slowed. While LWS fat depots and adipocyte sizes remained stable,
HWS chicks rapidly accumulated fat in CL and AB depots. Chicks predisposed to be anorexic or obese have different fat development patterns during the first two weeks post-hatch.

**Key words:** adipogenesis, adipose tissue depot, anorexic, obese, chicks, development

**Introduction**

Adipose tissue is the primary site in the body for storage of energy in the form of triglycerides [1]. Less is known about the regulation of adipose tissue physiology in birds than mammals, with most of the avian research conducted using chickens as the model organism. In humans and rodents, there are two main types of adipose tissue—the white and brown adipose tissue, whereas only white adipose tissue is found in chickens. In humans, white adipose tissue is mainly distributed beneath the skin (subcutaneous) and around the internal organs (visceral) [2]. In chickens and some other avian species, adipose tissue is mainly found in the abdominal and neck (clavicular) areas, as well as the subcutaneous regions [1, 3]. The cellular development and expansion of adipose tissue is a consequence of the balance between increases in cell number (hyperplasia) and cell size (hypertrophy). The progenitor cells, which eventually become mature adipocytes through adipogenesis, are regulated through hyperplasia [4], while hypertrophy is characterized by enhanced triglyceride storage and associated expansion of existing adipocytes [5]. In humans, white adipose tissue expands rapidly after birth through hyperplasia and hypertrophy [6]. During chicken embryonic adipose tissue development, preadipocyte hyperplasia dominates, followed by hypertrophy to establish immature adipocytes that can accumulate lipid droplets [7]. In both egg (layer) and meat-type (broiler) chickens, hyperplasia is induced in connective tissue in the neck and upper legs between embryonic days (E) 12 and 14, followed by slowing rates of hyperplasia until E18 [8]. Most adipocytes are unilocular by E14,
with a negligible number of multilocular cells (present during the early stages of adipocyte differentiation), indicating that chicken adipocytes undergo rapid maturation during embryonic development [8]. In humans, visceral adipose tissue accumulation is positively associated with the onset of metabolic diseases, and hypertrophy is predominant in adulthood obesity as it is observed in all overweight and obese individuals [5]. In chickens, adipocyte hyperplasia and hypertrophy increase with age, which is positively correlated with body mass and adipose tissue weight [9]. However, the magnitude of contribution of the two processes to the volume and weight of the distinct adipose tissue depots is likely different and not well understood. Though it is reported that abdominal fat development is more pronounced than subcutaneous or clavicular fat in broilers during the first two weeks post-hatch [10], in precocial avian species, there is a paucity of knowledge regarding metabolic characteristics of different fat depots.

We have previously reported on adipogenesis and lipid metabolism as they are affected by long-term genetic selection for low (LWS) or high (HWS) body weight in chickens. After long-term continuous selection for divergent body weight (BW) at 56 days of age from a common foundation population, there is a more than 10-fold difference in BW between the lines, accompanied by increased food intake in HWS compared with LWS chickens [11-14]. The LWS chickens are lean and anorexic, however, HWS chickens eat compulsively [15]. An earlier study reported that the LWS chicks have lower lipogenic but greater lipolytic activity in adipose tissue than HWS chicks, thus preventing excessive accumulation of body fat [16]. Furthermore, although hypertrophy plays a major role in fat deposition in adult chickens, caloric overconsumption induces hyperplasia in sexually mature LWS and HWS chickens [17]. Plasma non-esterified fatty acid concentrations are greater in 5 day-old LWS than HWS [18], and there is greater fatty acid oxidation efficiency and metabolic flexibility in the abdominal fat of 56-day
LWS chickens than their respective HWS counterparts [19]. Thus, while LWS chicks have a reduced capacity to accumulate adipose tissue that is likely related to a greater rate of metabolism, the underlying cellular mechanisms are unclear and the developmental regulation of these processes is unknown.

The objective of this study was to evaluate the morphological and molecular changes in depot-specific adipose tissue during the first two weeks post-hatch in chickens from lines selected for low and high BW. Although adipose tissue development under lean and obese states is comprehensively studied using rodent models, it is questionable whether the frequently used perigonadal depot in rodents can mimic the physiology of intra-abdominal/visceral adipose tissue in humans as 1) humans do not have an analogous perigonadal depot and 2) from rodents one can only harvest a limited quantity of mesenteric adipose tissue, which is considered the most anatomically and physiologically similar to human intra-abdominal adipose tissue [20]. Hence, our research using the body weight-selected chicken model, in which adipose tissue depots are anatomically similar to humans, will provide further insights on the basis for anatomical differences in adipose tissue expansion and mechanisms underlying the propensity to be lean or obese.

Materials and methods

Animals

All procedures were approved by the Virginia Tech Institutional Animal Care and Use Committee. The LWS and HWS chickens used in this study are the result of long-term selection for low or high body weight, respectively, at 56 days of age [12, 21] with details of the selection program described elsewhere [15, 22]. The parents of the chicks used in the study were at the
same age. After hatch, HWS and LWS lines were group-caged in a room at 32 ± 1 °C and 50 ± 5% relative humidity. Chicks had free access to water and a mash diet (21.5% crude protein and 3,000 kcal ME/kg). For BW, body composition, and fat depot weight measurements, six chicks were used per line. For plasma non-esterified fatty acid (NEFA) measurements, twelve chicks per line were used for the assay. For all the other experiments, five males and five females were used per line. Sexes were confirmed visually by gonadal inspection during dissection. On day of hatch (DOH), day 4 (D4), and D14 post-hatch, chicks were randomly selected from each line to be weighed and euthanized for tissue collection, as described below.

Body composition

Fat and lean masses were determined with a minispec LF90 NMR whole body composition analyzer (Bruker, MA, USA). The machine was calibrated with a bottle of 500 g of canola seeds before use as recommended by the manufacturer. Each chick was scanned twice and the average of the duplicates was used for data analysis. Prior to the measurements on DOH and D4, yolk sacs were completely removed from the euthanized birds by excision through the navel and weighed. This was necessary to eliminate the confounding effects of residual yolk nutrients on whole body composition.

Adipose tissue depot weights

Adipose tissue depots were weighed and weights were converted into a percentage of BW. The three depots included the abdominal (AB) (attached to the gizzard), clavicular (CL) (discrete mass above the clavicle) and subcutaneous (SQ) adipose tissue (after peeling back the skin above the cloaca and removing all exposed adipose tissue under the skin).

Adipose tissue histology
Adipose tissue samples were collected as described above. Samples were rinsed in phosphate-buffered saline, submerged in neutral-buffered formalin and incubated on a rocking platform at 4 °C overnight. Samples were dehydrated in a graded ethanol series, paraffin embedded, and sectioned at 5 μm and mounted on slides (one section was mounted per slide with two slides at least 200 μm apart per sample). Slides were stained with hematoxylin and eosin and images were captured (three on each section) with a Nikon Eclipse 80i microscope and DS-Ri1 color camera, and images analyzed using NIS-Elements Advanced Research Software (Nikon). Adipocytes were treated as binary objects with the restriction that measurements must exceed 100 μm². The area and diameter of every adipocyte within the field of an image were measured under 20x magnification. The adipocyte size distribution was also determined for each group.

**Total RNA isolation and real-time PCR**

Adipose tissue samples were collected and submerged in RNAlater (Qiagen, CA, USA). Tissues were homogenized in 1 mL Tri-Reagent (Molecular Research Center, TR 118) using 5 mm stainless steel beads (Qiagen) and a Tissue Lyser II (Qiagen) for 2 × 2 min at 25 Hz. The manufacturer’s instructions were followed to separate total RNA and after the step of addition to 100% ethanol, the RNA and ethanol mixture was transferred to spin columns and further purified with the optional RNase-free DNase I from Direct-zol RNA kits (Zymo Research, CA, USA). A high Capacity cDNA Reverse Transcription Kit (Applied Biosystems, CA, USA) was used to synthesize single-stranded cDNA. Primers for real-time PCR were designed with Primer Express 3.0 software (Applied Biosystems), and validated to have similar (within 5% of reference gene) amplification efficiency before use (Table 3.1). Real-time PCR was performed as we described [10].

*Plasma non-esterified fatty acids (NEFAs)*
The procedure was the same as described in our previous study [18]. Briefly, approximately 200 μL of blood was collected from the trunk of each chick via Microvette® capillary blood collection tubes (Sarstedt, Germany) immediately following euthanasia and decapitation. After collection, samples were centrifuged at 2,000 x g at room temperature to isolate plasma, which was then placed on ice and aliquoted. Plasma NEFA concentrations were measured with a NEFA-HR2 kit (FUJIFILM Wako Diagnostics, CA, USA) according to the manufacturer’s instructions. Absorbance was measured at 550 nm using an Infinite M200 Pro multi-mode plate reader (Tecan, Switzerland). 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.

**Statistical analysis**

Analysis of variance (ANOVA) was performed for BW, body composition, adipose tissue depot weight, percent weight, adipocyte area and diameter, relative quantity (RQ) value, and NEFAs using the Fit Model platform of JMP Pro 13 (SAS Institute, NC, USA). Correlations between yolk sac weight and body weight, lean and fat mass on DOH and D4 were calculated using the Fit Y by X procedure of JMP Pro 13. 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 [23]. The average of SQ fat on DOH from the LWS was used as the calibrator sample. The fold difference (RQ) was calculated as $2^{-\Delta\Delta CT}$. Effects involving sex were not significant, thus sex was excluded from the statistical model. The statistical model included the main effects of adipose tissue depot, day of sampling (age), line, and their interactions (only age and line for BW and body composition data). Tukey’s test was used post hoc to separate the means. All data
are presented as least squares means ± standard error of mean (SEM). Differences were
considered significant at $P < 0.05$.

Results

For all results, only the highest-order significant effect for each trait will be discussed. There was
a two-way interaction between line and age on BW, where HWS chicks were heavier than LWS
at all of the sampling days ($P < 0.0001$; Figure 3.1).

Body composition

Body composition data are summarized in Table 3.2. Yolk sac weight (YSW) as a percentage of
BW decreased from DOH to D4 ($P < 0.0001$). The absolute fat mass values on DOH were too
low to be determined accurately by the instrument, hence the absolute and relative fat mass data
on DOH were omitted from the analysis. Relative lean (lean%) masses as a percentage of BW
were greater in HWS than LWS ($P < 0.0001$).

There were line by age interactions on absolute YSW ($P = 0.0254$; Figure 3.2), absolute fat and
lean mass and fat% ($P < 0.0001$; Figure 3.3). HWS chicks had the greatest YSW on DOH,
followed by that of the LWS at the same age, whereas by D4 YSW had decreased to a similar
amount in both lines (Figure 3.2). Lean mass increased at each age in HWS and was greater in
HWS than LWS at each age, whereas in LWS chicks, lean mass did not change between DOH
and D4 but was greater at D14 than D4 (Figure 3.3A). Similarly, fat mass, both absolute (Figure
3.3B) and as a percentage of BW (Figure 3.3C), was greater in HWS than LWS at D4 and D14,
and increased to D14 at a greater magnitude in HWS than LWS chicks.
In addition, the relationships between absolute YSW and BW, fat mass and lean mass were also determined. There were significant correlations between YSW and BW, as well as between YSW and lean mass on both DOH (\(r = 0.83, P = 0.0008\) and \(r = 0.755, P = 0.0045\), respectively) and D4 (\(r = 0.744, P = 0.0055\) and \(r = 0.721, P = 0.0081\), respectively) regardless of line. A correlation between YSW and fat mass on D4 post-hatch (\(r = 0.812, P = 0.0013\)) was also observed regardless of line. However, within each line, the only correlation was between YSW and BW in LWS chicks on DOH (\(r = 0.847, P = 0.0332\); Figure 3.4A), while on D4 it approached significance (\(r = 0.809, P = 0.0512\); Figure 3.4C). There were also linear relationships between DOH YSW and D4 and D14 BW (\(P = 0.0071\), Figure 3.5A and \(P = 0.0073\), Figure 3.5E, respectively), fat mass (\(P = 0.0301\), Figure 3.5B and \(P = 0.0076\), Figure 3.5F, respectively), as well as lean mass (\(P = 0.0063\), Figure 3.5C and \(P = 0.0060\), Figure 3.5G, respectively) regardless of line. In HWS chicks, there was a linear relationship between DOH YSW and D4 fat\% (\(P = 0.0171\), Figure 3.5D), while no such association was observed in LWS. Moreover, relationships between D4 YSW and D14 BW (\(P = 0.0051\), Figure 3.5H), fat mass (\(P = 0.0052\), Figure 3.5I) and lean mass (\(P = 0.0069\), Figure 3.5J) were observed regardless of line.

**Adipose tissue depot weights**

Results for adipose tissue depot weights are summarized in Table 3.3. There was a three-way interaction of age, adipose tissue depot, and line on absolute depot weights (\(P < 0.0001\); Figure 3.6A). Tissue weight was greatest in CL, intermediate in AB, and lowest in SQ of HWS at D14. For other combinations, weights were either similar or lower than that of SQ in HWS at D14. There were no differences among depots and ages within LWS chicks.
There were two-way interactions of age and adipose tissue depot, age and line, as well as adipose tissue depot and line for relative depot weights ($P < 0.0001$, $P < 0.0001$, and $P = 0.0007$, respectively; Figure 3.6B-D). SQ% at DOH and CL% at D14 were greater than other age and depot combinations. Relative depot weights were greater in HWS than other age and line combinations. SQ% in LWS was similar to CL% in HWS, which was greater than the relative weights in other adipose tissue depot and line combinations.

**Adipose tissue histology**

Results for adipocyte measurements are summarized in Table 3.4. There were interactions of age and depot, age and line, and depot and line on adipocyte areas ($P = 0.0003$, $P < 0.0001$ and $P = 0.01$, respectively; Figure 3.8A, B and C, respectively) as well as adipocyte diameters ($P = 0.005$, $P < 0.0001$ and $P = 0.007$, respectively; Figure 3.8D, E and F, respectively). Adipocyte area (Figure 3.8A) and diameter (Figure 3.8D) of SQ fat at DOH were greater than in other depots at all ages. For both traits, in all depots, there was a decrease from DOH to D14.

Adipocyte areas (Figure 3.8B) and diameters (Figure 3.8E) were greater in both lines at DOH than other ages as well as in HWS at D14 than other age and line combinations. Adipocyte area (Figure 3.8C) and diameter (Figure 3.8F) were similar among depots in LWS, whereas in HWS they were greatest in the SQ fat and lowest in the AB fat, and greater in the HWS SQ fat than other depot and line combinations.

Adipocyte size distributions are shown in Figure 3.9. In terms of the distribution of adipocyte diameters, more than 94%, 95% and 98% of adipocytes were 20 to 45 μm (Figure 3.9A), 15 to 40 μm (Figure 3.9B) and 15 to 40 μm (Figure 3.9C) in the SQ, CL and AB fat, respectively, of LWS chicks at DOH. More than 99% (95% in AB fat at D14) of adipocytes were 10 to 30 μm in the SQ fat of LWS at D4 and D14, and 10 to 25 μm in the AB fat of LWS at D4 and D14. The
distribution was similar in the SQ (Figure 3.9D) and CL (Figure 3.9E) fat of HWS from DOH to D14. On D4, the diameter of more than 98% of adipocytes in the AB fat of HWS was smaller than 25 μm, whereas the diameter of more than 45% of adipocytes was larger than 25 μm at D14 (Figure 3.9F).

mRNA abundance in adipose tissue

There were no two- or three-way interactions for adipose triglyceride lipase (ATGL) mRNA. Therefore, only the main effects of age, depot and line on ATGL expression are presented. The mRNA abundance of ATGL increased from DOH to D4, and declined from D4 to D14 to the same level as on DOH. Among the three depots, ATGL expression was greater in CL than AB, while expression in SQ did not differ from the other two depots. LWS chicks expressed more ATGL than the HWS chicks.

Interactions of age and adipose tissue depot. While the mRNA abundance of 1-acylglycerol-3-phosphate O-acyltransferase 2 (AGPAT2; Figure 3.10A) in SQ increased from DOH to D14, that of CCAAT/enhancer-binding protein alpha (C/EBPα; Figure 3.10B), fatty acid binding protein 4 (FABP4; Figure 3.10C) and neuropeptide Y receptor 2 (NPYR2; Figure 3.10E) followed the same pattern from DOH to D14 in SQ, where expression increased from DOH to D4, then declined back to the same level as DOH on D14. Though the expression of lipoprotein lipase (LPL; Figure 3.10D) and sterol regulatory element-binding transcription factor 1 (SREBP1; Figure 3.10F) in SQ also increased during this period, their expression was similar between D4 and D14. In the CL depot, expression of AGPAT2 declined from DOH to D4, while FABP4, LPL and SREBP1 increased and C/EBPα and NPYR2 remained similar. From D4 to D14, expression of AGPAT2, FABP4 and NPYR2 decreased in CL, SREBP1 increased, while C/EBPα and LPL remained similar. AGPAT2 and FABP4 decreased from DOH to D14 in the AB depot, while
SREBP1 increased to D14. NPYR2 increased in AB from DOH to D4, then decreased about 8-fold at D14, to an amount that was even lower than on DOH.

*Interactions of age and line.* In both lines, diglyceride acyltransferase (DGAT2) was similar at DOH and D4, after which it increased from D4 to D14, with the magnitude of increase from D4 to D14 greater in HWS than LWS (Figure 3.11A). Although FABP4, LPL, NPYR2 and SREBP1 all increased from DOH to D4 in LWS, only LPL and SREBP1 followed the same expression pattern in HWS as LWS, while FABP4 and NPYR2 were similar at DOH and D4 in HWS (Figure 3.11B-E). From D4 to D14, FABP4 and NPYR2 decreased in both lines, whereas the expression of LPL and SREBP1 was the same in LWS, but decreased and increased in the HWS chicks, respectively. When comparing the mRNA abundance at each time point, more FABP4 and NPYR2 were expressed in LWS than HWS on D4, while more LPL and SREBP1 were expressed in HWS than LWS on D4 and D14, respectively. At other time points, the mRNA abundance of these genes was the same between lines.

*Interactions of adipose tissue depot and line.* AGPAT2 (Figure 3.12A) and SREBP1 (Figure 3.12C) mRNAs were expressed similarly among all three depots in LWS. In contrast, in HWS, AGPAT2 was greater in CL than AB and SREBP1 was greater in CL than SQ and AB. Although the expression of NPYR2 (Figure 3.12B) was similar among all depots in HWS, expression was greater in AB than SQ and CL in LWS. When comparing the mRNA abundance within the same depot between lines, LWS had greater amounts of AGPAT2 and NPYR2 in AB than HWS, whereas HWS had greater expression of SREBP1 in CL than LWS chicks.

*Interactions of age, adipose tissue depot, and line.* Acyl-CoA dehydrogenase, long chain (ACADL) had greater expression in HWS CL at D14 than in all other line, depot, and age
combinations (Figure 3.14A). In other depots in both lines, ACADL mRNA was similar or reduced at D14 as compared to D4. C/EBPβ mRNA was greatest at D4 in all depots in LWS chicks, but only in CL in the HWS (Figure 3.14B). In other depots in HWS, C/EBPβ expression was similar among the three ages, while in LWS, expression in all depots was similar between DOH and D14. The mRNA abundance of neuropeptide Y (NPY) was also greater at D4 than other ages within all depots in LWS chicks, with similar expression between DOH and D14 (Figure 3.14C). In HWS, NPY expression was similar among ages in SQ and CL, and greater at DOH than D14 in AB. Expression of NPY at D4 in both the CL and AB depots was greater in LWS than HWS chicks. Peroxisome proliferator-activated receptor gamma (PPARγ) mRNA was greater at D4 than other ages in the CL and AB of LWS chicks (Figure 3.14D). In HWS chicks, expression was greater at D4 than DOH in CL and D4 than DOH in SQ. In LWS chicks, there was a more accentuated difference between D4 and D14 PPARγ expression in CL and AB depots than in HWS chicks.

Interaction of age and genetic line on plasma NEFA concentrations

There was an interaction of age and genetic line (P < 0.0001), where plasma NEFAs were similar between the lines at DOH but greater in LWS than HWS at D4 and D14 (Figure 3.14).

Discussion

Development from DOH to D4 post-hatch

Yolk, which is predominantly comprised of lipids, is the major energy source during embryonic development of the chick and remains a primary energy source during the first few days post-hatch. It accounts for about 20% of the total body weight of chicks at hatch [24] and greater than 50% of yolk absorption occurs during the first 48 hours post-hatch [25], with the residuals being
essentially absorbed within four days post-hatch [26]. Our findings here were consistent with these previous reports. During the first four days post-hatch, there was a dramatic decline of both absolute and relative YSW regardless of line, indicating a rapid utilization of yolk during this developmental stage. The positive regressions between DOH YSW and D4 and D14 BW, fat mass and lean mass may suggest that higher yolk reserves at hatch provide an important energy source for early post-hatch development, and thus enhanced growth performance. When analyses were performed within each line, the correlation between YSW and BW was observed in LWS but not HWS chicks, while on DOH YSW of HWS chicks had a negative regression with D4 fat mass as a percentage of BW. Intrigued by such results, we further analyzed the relationships between D4 YSW and D4 and D14 BW, fat mass and lean mass, which again showed positive correlations and regressions regardless of line. Upon within-line analyses, there was a positive correlation between D4 YSW and D4 BW only in LWS chicks. Turro-Vincent et al. (1994) reported that the digestive tract is better developed in the HWS than LWS chicks during the early post-hatch stage and thus the HWS chicks are more efficient in carbohydrate utilization upon feeding while the LWS chicks are more capable of lipid digestion [27]. Bhanja et al. (2009) reported that broilers with delayed access to feed post-hatch have slower yolk absorption but have higher lipid retention from the yolk [28]. In support of such results, our findings indicate that the anorexic LWS and hyperphagic HWS chicks may have different yolk utilization mechanisms. With the presence of feed upon hatch, the HWS chicks may depend less on nutrients from the yolk and rapidly transit to diets rich in carbohydrates to facilitate early development. In contrast, due to the delayed development and loss of appetite, the LWS chicks are more dependent on nutrients provided by yolk residuals.
During the first four days post-hatch, there was little change in BW, lean mass, or lean mass as a percentage of BW of LWS chicks (data not shown). Although the lean mass as a percent of BW did not change in HWS chicks, their BW and absolute lean mass increased substantially during this period. These results together with the transition from undetectable to detectable fat mass during the first 4 days post-hatch demonstrate that both LWS and HWS accumulated body fat from DOH to D4, with much greater accumulation in HWS to promote the overall increase in BW occurring during that time.

After E12, there is rapid lipid deposition in SQ in chicken embryos, and at hatch, it is mobilized, providing extra energy to facilitate the hatching process [8]. In broilers, from DOH to D4 post-hatch, there is a reduction in SQ as a percent of BW with unchanged SQ weight [29]. This is consistent with the observation in LWS during the first 4 days post-hatch, whereas neither SQ weight as a proportion of body weight nor SQ weight changed in HWS. Also in LWS, the average adipocyte diameter was reduced in all depots from DOH to D4, whereas it remained about the same in HWS. This suggests that the LWS chicks, due to their tendency to be hypophagic, may need to utilize more fat reserves and/or replenish the reservoir slower in order to survive during the early post-hatch period.

Indeed, our results show that LWS chicks overall expressed greater amounts of ATGL, FABP4, NPY and NPYR2 mRNA than HWS, and ACADL and C/EBPβ in SQ fat from DOH to D4. In contrast, the HWS had a surge in LPL expression regardless of depots, and in CL, only HWS chicks increased expression of C/EBPβ in their adipose tissue during the first four days post-hatch. Both ATGL and hormone sensitive lipase (HSL) are the major enzymes involved in lipolysis, accounting for over 95% of lipolytic activity in murine adipose tissue [30]. However, no orthologue of HSL has been identified in the chicken genome [31], suggesting that ATGL
may be the primary enzyme responsible for lipolysis in chickens. Because the mobilization of yolk during embryonic development does not require ATGL, its high expression after hatch is thought to exclusively reflect the utilization of fat reserves from adipose tissue [32]. FABP4 serves as a marker of mature adipocytes and in rodents it prohibits lipogenesis through negative feedback on PPARγ and enhances lipolysis through activation of HSL [33]. It was also observed in chickens that fat males had less FABP4 mRNA in the AB depot than lean ones at two weeks post-hatch, which is also postulated to be related to the enhanced lipolysis induced by greater FABP4 expression, although HSL has not been identified in chickens [34]. ACADL is an enzyme that catalyzes the first step of the β-oxidation of long chain fatty acids in the mitochondria [35]. Thus, expression patterns of ATGL, FABP4 and ACADL reflect greater catabolic activity in the adipose tissue of LWS chicks, and more potent fatty acid oxidation in the SQ depot of LWS than HWS chicks. This observation is further confirmed by the plasma NEFA change during the first four days, where the concentrations declined significantly in HWS but not LWS, reflecting reduced lipolytic activity in the HWS adipose tissue. These results here are consistent with a previous report showing that juvenile (56-65 days old) LWS chicks have more metabolic flexibility and fatty acid oxidation efficiency in their adipose tissue than HWS [19].

As a transcription factor, C/EBPβ plays essential roles during adipogenesis, with its functions best understood in mammalian models. It facilitates mitotic clonal expansion during early adipogenesis, which is a prerequisite for its activation of PPARγ and C/EBPα leading to the terminal transition from a preadipocyte to a mature adipocyte [36, 37]. Increased expression of C/EBPβ in the SQ of LWS may indicate that LWS chicks compensated for the mobilization of SQ reservoirs through a potent stimulation of adipogenesis. However, the replenishment of fat reserves may not be sufficient or rapid enough to offset rates of utilization, leading to the overall
reduction in SQ% in the LWS chicks. To the contrary, increased $C/EBP\beta$ expression in the CL of HWS, together with the up-regulation of $LPL$, which encodes the “gatekeeper” enzyme to facilitate lipid deposition in adipocytes [38], collectively suggest that HWS chicks replenished their CL fat reserves through a combination of hyperplasia and hypertrophy to maintain CL% and size. On the other hand, the smaller adipocytes in LWS chicks reflect reduced lipid deposition capacity compared to the HWS. The reduction in AB adipocyte size exclusively in the LWS may indicate that there is mobilization from the AB depot as well, although AB fat is negligible in LWS at hatch and changes very little with age during the first two weeks post-hatch. Interestingly, gene expression changes in AB fat are suggestive of an induction of adipogenesis in LWS chicks, specifically the greater expression of $C/EBP\beta$ and $PPAR\gamma$ in LWS than HWS, despite that the depot does not increase in weight with age in LWS chicks.

NPY is a potent orexigenic factor in both mammals [39-41] and chickens [42]. NPY also promotes adipogenesis in adipocyte progenitor cells in mammals [43] and chickens [44, 45] in vitro, most likely through NPYR2 [43]. In situ hybridization further confirmed the expression of $NPY$ in AB of 14-day old LWS (Figure A.1) and HWS (Figure A.2). Both $NPY$ and $NPYR2$ expression sharply increased in LWS from DOH to D4 regardless of depot, while neither changed in the HWS chicks. This suggests that NPY, together with transcription factors, promotes adipogenesis in LWS during the early post-hatch period to help replenish and offset the high consumption of fat reserves. However, due to the low lipid deposition capacity, the new adipocytes are relatively small and not as receptive to lipid accumulation as in HWS chicks.

**Development from D4 to D14 post-hatch**

From D4 to D14, both lines increased in BW, which was attributable to both fat and lean mass deposition. Body fat percentage increased more than 10% regardless of line, while lean
percentage did not change, indicating that fat deposition was the predominant factor that
promoted BW increase during this developmental phase. However, not one of the three adipose
tissue depots that we measured changed in absolute or relative mass, nor was there any change in
adipocyte size in the LWS chicks. It should be noted that although adipose tissue is the primary
site for fat deposition, the three major depots measured here together with sartorial and
mesenteric depots contribute about 20% to total body fat mass, while the rest of the carcass (e.g.
other adipose depots, intestine and muscles) and skeleton contribute about 55%, and the liver,
skin and feathers contribute about 20% in the broilers [1]. Therefore, the results herein indicate
that other fat-rich organs such as the brain, which contains over 60% fat [46, 47], and liver and
skeletal muscle, which are essential for ectopic fat deposition [48], may have contributed more to
the overall fat mass increase in LWS chicks.

In contrast, mass increased in HWS chicks in all three depots from D4 to D14, although the
adipocytes only increased in size in AB, but not CL or SQ. In 14-day old broilers, the adipocyte
size difference among depots was CL > SQ > AB [10], whereas adipocytes in HWS at the same
age showed no difference among depots. Such differences, together with the observation that the
mRNA expression of ACADL decreased in SQ while it increased in CL from D4 to D14 in HWS
chicks, suggests that HWS chicks mobilized less of their energy reserves in SQ but catabolized
more in CL than broilers during this period of development. However, thereafter at 56 days of
age, the adipocyte size difference among depots in male HWS birds is CL > AB > SQ due to
continuous lipid deposition in CL and AB depots as they approach sexual maturity [19]. From
D4 to D14, decreased expression of C/EBPβ, PPARγ and NPY mRNAs was observed in almost
all depots of LWS chicks, while except for C/EBPβ in CL, expression remained the same in all
other depots of HWS. These results, together with the increased expression of SREBP1, a master
regulator of fatty acid synthesis [49], and $DGAT2$, which catalyzes the terminal step of triacylglycerol synthesis [50], suggest that the general expansion of adipose depots in HWS was likely a result of both hyperplasia and hypertrophy, the same as in broilers from D4 to D14 post-hatch [10]. Moreover, the increase in CL% but not size and the increase in AB% with enlarged adipocytes suggests that the expansion of CL depot is mainly through hyperplasia whereas AB depot expansion occurs predominantly through hypertrophy in HWS chicks.

In LWS chicks, most genes involved in adipogenesis had reduced expression, while the maintenance of depot mass and adipocyte size suggest that there was a balance between utilization and replenishment, indicating a slower catabolism of fat reserves with sufficient replenishment of the reservoir from D4 to D14 post-hatch. This balance was likely supported by reduced fatty acid oxidation ($ACADL$) and increased fatty acid synthesis ($DGAT2$). It is worth noting that throughout the first two weeks post-hatch, although the genes were differentially expressed between lines, most mRNAs measured in our study were expressed similarly between LWS and HWS within the same depot and/or at the same age. This begs the question—why under such conditions are the overall fat masses and adipocyte sizes smaller in LWS than HWS? Except for the more potent catabolism, another explanation could be that cellular processes like apoptosis and necrosis somehow halted adipogenesis either at an early stage to prohibit the maturation of adipocytes, or at a later stage to limit the deposition of lipids in the adipocytes in LWS chicks.

We conclude from the data that LWS and HWS chicks have dramatic differences in their physiological development during the first two weeks of life, with the HWS having a much more accentuated BW gain and accumulation of adipose tissue independent of the yolk residuals during this time. From DOH to D4, there is rapid absorption of yolk sac and intense mobilization
of fat reserves accompanied by potent hyperplasia and hypertrophy to replenish the reservoirs of energy. LWS chicks are mainly dependent on hyperplasia in sacrifice of increasing pre-existing adipocyte size due to their lower cellular lipid deposition capacity, whereas HWS balance utilization and replenishment through hyperplasia and hypertrophy to maintain adipocyte size and support increases in depot mass. From D4 to D14, rates of adipose tissue catabolism and adipogenesis decelerate. While LWS have balanced mobilization and lipid replenishment, HWS rapidly accumulate fat in their clavicular and abdominal regions.

**Perspectives and significance**

Our results indicate that the slower development of LWS, which are predisposed to be anorexic, limited their food intake and/or utilization of exogenous feed for early development and thus resulted in the higher dependence on yolk and intensive catabolism of the body fat reserves, whereas the HWS are capable of utilizing feed for growth and deposit lipid efficiently to promote the expansion of adipose depots. These results provide further insights on the understanding of different physiological mechanisms underlying early depot-specific adipose tissue development in chickens predisposed to be anorexic or obese. These data may also shed light on the mechanism of adipose tissue expansion that underlies a propensity to be lean or obese in humans due to the anatomical similarities between the two species. It should be noted that our study only focused on the major adipose tissue depots in chickens, while fat is also a critical component of tissues including brain, liver and skeleton muscle. Therefore, the depot changes may not necessarily reflect the overall changes in body fat mass. Further study of the fat mass changes in other tissues may compliment and provide better understanding of contributions from each component to the overall body fat mass during early development. Meanwhile, further research at the cellular level could identify differences between these two lines at different cell
developmental stages (progenitor versus adipocyte) and whether apoptosis/necrosis plays an inhibitory role in adipose tissue expansion in LWS.

Acknowledgements

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References

Table 3.1. Primers used for real-time PCR.

<table>
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<th>Accession No.</th>
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<tr>
<td>ACADL</td>
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<td>AGPAT2</td>
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<tr>
<td>C/EBPβ</td>
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1Gene abbreviations: Acyl-CoA dehydrogenase, long chain: ACADL; 1-acylglycerol-3-phosphate O-acyltransferase 2: AGPAT2; adipose triglyceride lipase: ATGL; CCAAT/enhancer-binding protein alpha and beta: C/EBPα and C/EBPβ, respectively; diacylglycerol O-acyltransferase 2: DGAT2; fatty acid binding protein 4: FABP4; lipoprotein lipase: LPL; neuropeptide Y: NPY; NPY receptor 2: NPYR2; peroxisome proliferator-activated receptor gamma: PPARγ; sterol regulatory element-binding transcription factor 1: SREBP1.
Table 3.2. Body composition of body weight-selected line chicks during the first two weeks post-hatch.

<table>
<thead>
<tr>
<th>Effect</th>
<th>YSW (g)</th>
<th>YSW (%BW)</th>
<th>Fat mass (g)</th>
<th>Fat mass</th>
<th>Lean mass (g)</th>
<th>Lean mass (%BW)</th>
</tr>
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<tbody>
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<td>Age</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DOH</td>
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<td>12.79</td>
<td>-</td>
<td>-</td>
<td>24.30&lt;sup&gt;c&lt;/sup&gt;</td>
<td>62.25</td>
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<tr>
<td>D4</td>
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<td>0.65</td>
<td>5.06</td>
<td>6.98</td>
<td>45.03&lt;sup&gt;b&lt;/sup&gt;</td>
<td>61.98</td>
</tr>
<tr>
<td>D14</td>
<td>-</td>
<td>-</td>
<td>42.96</td>
<td>20.52</td>
<td>125.86&lt;sup&gt;a&lt;/sup&gt;</td>
<td>60.23</td>
</tr>
<tr>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LWS</td>
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<td>13.03</td>
<td>2.73</td>
<td>7.32</td>
<td>15.85</td>
<td>52.93</td>
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<tr>
<td>HWS</td>
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<td>24.01</td>
<td>13.75</td>
<td>65.06</td>
<td>61.49</td>
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<tr>
<td>SEM</td>
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<td>0.67</td>
<td>0.55</td>
<td>0.33</td>
<td>0.79</td>
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Effects of age (day of hatch: DOH; day 4 post-hatch: D4; and day 14 post-hatch: D14), line (low- and high-body weight-selected lines; LWS and HWS, respectively) and the interaction between age (A) and line (L) on absolute yolk sac weight (YSW), lean and fat mass and as a percentage of body weight (%BW). Values represent least squares means and pooled SEM with associated P-values for each effect (n = 6). Unique superscripts within an effect denote differences at P < 0.05. (-) denotes that there was no comparison for the amount of fat on DOH because fat percentages were too low in each line to be determined accurately by the instrument and on D14, there was no yolk sac remaining.
Table 3.3. Adipose tissue depot weights.

<table>
<thead>
<tr>
<th>Effect</th>
<th>Weights (g)</th>
<th>Weights (%BW)</th>
</tr>
</thead>
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<tr>
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<tr>
<td>DOH</td>
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<tr>
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<tr>
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<td>SQ</td>
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<tr>
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<tr>
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*Effects of age (day of hatch: DOH; day 4 post-hatch: D4; and day 14 post-hatch: D14), adipose tissue depot (subcutaneous: SQ; clavicular: CL; and abdominal: AB), line (low- and high-body weight-selected lines; LWS and HWS, respectively), and the interactions between age (A), depot (D), and line (L) on adipose tissue weights and weights as a percentage of body weight (%BW). Values represent least squares means and pooled SEM with associated P-values for each effect (n = 6). Unique superscripts within an effect are significantly different at P < 0.05.*
Table 3.4. Adipocyte area and diameter.

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<tr>
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<td>22.71&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>&lt;0.0001</td>
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†Effects of age (day of hatch: DOH; day 4 post-hatch: D4; and day 14 post-hatch: D14), adipose tissue depot (subcutaneous: SQ; clavicular: CL; and abdominal: AB), line (low- and high-body weight-selected lines; LWS and HWS, respectively) and the interactions between age (A), depot (D), and line (L) on adipocyte area and diameter. Values represent least squares means and pooled SEM with associated P-values for each effect (n = 10). Unique superscripts within an effect reflect differences at P < 0.05.
Table 3.5. Adipose tissue mRNA abundance.

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†Effects of age (day of hatch: DOH; day 4 post-hatch: D4; and day 14 post-hatch: D14), adipose tissue depot (subcutaneous: SQ; clavicular: CL; and abdominal: AB), line (low- and high-body weight-selected lines; LWS and HWS, respectively) and the two-way interactions between age (A) and depot (D), age (A) and line (L), depot (D) and line (L), and the three-way interactions among age (A), depot (D) and line (L). Values represent least squares means and pooled SEM with associated P-values for each effect (n = 10).
Unique superscripts within an effect are significantly different at $P < 0.05$. Abbreviations: Acyl-CoA dehydrogenase, long chain: ACADL; 1-acylglycerol-3-phosphate O-acyltransferase 2: AGPAT2; Adipose triglyceride lipase: ATGL; CCAAT/enhancer-binding protein alpha and beta: C/EBPα and C/EBPβ, respectively; Diglyceride acyltransferase 2: DGAT2; Fatty acid binding protein 4: FABP4; Lipoprotein lipase: LPL; Neuropeptide Y: NPY; NPY receptor sub-type 2: NPYR2; Peroxisome proliferator-activated receptor gamma: PPARγ; Sterol regulatory element-binding transcription factor 1: SREBP1.
Figure 3.1. Body weights of low (LWS) and high (HWS) body weight-selected line chicks on day of hatch (DOH), day 4 (D4) and day 14 (D14) post-hatch. Means bearing unique letters differ ($P < 0.05$; Tukey’s test).
Figure 3.2. Yolk sac weights of low (LWS) and high (HWS) body weight-selected line chicks on day of hatch (DOH) and day 4 post-hatch (D4). Tukey’s test was performed to separate the significant two-way interactions between age and line. Means bearing unique letters differ ($P < 0.05$).
Figure 3.3. Body composition of low (LWS) and high (HWS) body weight-selected line chicks on day of hatch (DOH), and days 4 (D4) and 14 (D14) post-hatch. Tukey’s test was used to separate the significant two-way interactions between age and line on lean mass (A), fat mass (B) and fat mass expressed as a percentage of BW (C). Means bearing unique letters differ ($P < 0.05$).
Figure 3.4. Correlations between yolk sac weight and body weight, lean mass and fat mass on day of hatch (DOH; A-B) and day 4 post-hatch (D4; C-E) in high (HWS) and low (LWS) body weight-selected line chicks. *$P < 0.05$. 
Figure 3.5. Regressions between yolk sac weight and body weight. Regressions between day of hatch yolk sac weight and day 4 post-hatch body weight (A), fat mass (B) and lean mass (C) regardless of line and regression between day of hatch yolk sac weight and day 4 post-hatch fat mass in percentage of body weight (fat%) in high (HWS) and low (LWS) body weight-selected line chicks (D). Regressions between day of hatch yolk sac weight and day 14 post-hatch body weight (E), fat mass (F) and lean mass (G) regardless of line. Regressions between day 4 post-hatch yolk sac weight and day 14 post-hatch body weight (H), fat mass (I) and lean mass (J). Red dots represent LWS chicks and black dots represent HWS chicks (*P < 0.05).
Figure 3.6. Adipose tissue depot absolute weight and weight on a body weight basis (%BW) in subcutaneous (SQ), clavicular (CL), and abdominal (AB) depots on the day of hatch (DOH), and days 4 (D4) and 14 (D14) post-hatch in low (LWS) and high (HWS) body weight-selected line chicks. Tukey’s test was used to separate the significant three-way interactions of age, line and depot (A), and two-way interactions between age and adipose tissue depot (B), age and line (C), and adipose tissue depot and line (D). Means bearing unique letters differ ($P < 0.05$).
Figure 3.7. Representative images from histological evaluation of subcutaneous (SQ), clavicular (CL), and abdominal (AB) adipose tissue depots in low (LWS) and high (HWS) body weight-selected lines of chicks on day of hatch (A), day 4 post-hatch (B) and day 14 post-hatch (C). Scale bar = 50 μm. Sections were hematoxylin and eosin-stained with images captured by a Nikon Eclipse 80i microscope and DS-Ri1 color camera.
Figure 3.8. Adipocyte area and diameter in subcutaneous (SQ), clavicular (CL), and abdominal (AB) adipose tissue depots of low (LWS) and high (HWS) body weight-selected lines of chicks on day of hatch (DOH) and days 4 (D4) and 14 (D14) post-hatch. Tukey’s test was performed to separate the interactions between age and adipose tissue depot (A, D), age and line (B, E), and adipose tissue depot and line (C, F). Means bearing unique letters differ ($P < 0.05$).
Figure 3.9. Adipocyte size distribution in subcutaneous (SQ; A), clavicular (CL; B), and abdominal (AB; C) adipose tissue depots of low body weight-selected (LWS), and SQ (D), CL (E), and AB (F) depots of high body weight-selected (HWS) line chicks on day of hatch (DOH) and days 4 (D4) and 14 (D14) post-hatch.
Figure 3.10. Interactions of age and adipose tissue depot on the mRNA abundance of AGPAT2 (A), C/EBPα (B), FABP4 (C), LPL (D), NPYR2 (E), and SREBP1 (F) in subcutaneous (SQ), clavicular (CL), and abdominal (AB) adipose tissue depots on day of hatch (DOH) and days 4 (D4) and 14 (D14) post-hatch. Tukey’s test was used to separate the interactions between age and adipose tissue depot. Means bearing unique letters differ ($P < 0.05$). See main text for description of gene abbreviations.
Figure 3.11. Interactions of age and line on the mRNA abundance of \textit{DGAT2} (A), \textit{FABP4} (B), \textit{LPL} (C), \textit{NPYR2} (D), and \textit{SREBP1} (E) on day of hatch (DOH) and days 4 (D4) and 14 (D14) post-hatch in low (LWS) and high (HWS) body weight lines of chicks. Tukey’s test was used to separate the interactions between age and line. Means bearing unique letters differ ($P < 0.05$). See main text for description of gene abbreviations.
Figure 3.12. Interactions of genetic line and adipose tissue depot on the mRNA abundance of AGPAT2 (A), NPYR2 (B) and SREBP1 (C) in subcutaneous (SQ), clavicular (CL), and abdominal (AB) depot of low (LWS) and high (HWS) body weight lines of chicks. Tukey’s test was used to separate the interactions between adipose depot and line. Means bearing unique letters differ ($P < 0.05$).
Figure 3.13. Interactions of age, adipose tissue depot, and genetic line on the mRNA abundance of ACADL (A), C/EBPβ (B), NPY (C) and PPARγ (D) in subcutaneous (SQ), clavicular (CL), and abdominal (AB) depot on day of hatch (DOH) and days 4 (D4) and 14 (D14) post-hatch. Tukey’s test was used to separate the three-way interactions among age, adipose depot and line. Means bearing unique letters differ ($P < 0.05$).
Figure 3.14. Plasma non-esterified fatty acid (NEFA) concentration in low (LWS) and high (HWS) body weight-selected line chicks on day of hatch (DOH), and days 4 (D4) and 14 (D14) post-hatch. Tukey’s test was used to separate the significant two-way interaction between age and line. Means bearing unique letters differ ($P < 0.05$).
Chapter 4: Adipogenic, metabolic, and apoptotic marker mRNA in cellular fractions of adipose tissue from chickens predisposed to be anorexic or obese

As published in Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology in 2019 (238:110555)

Abstract

The body weight-selected lines of chickens are a model for understanding factors that predispose an individual to anorexia or obesity. The high body weight-selected (HWS) individuals are compulsive eaters that become obese whereas the low body weight-selected (LWS) are relatively lean and hypophagic. The objective of this study was to measure gene expression of various preadipocyte, proliferation, metabolic, and apoptotic markers in the stromal-vascular fraction and adipocytes from LWS and HWS adipose tissue. Although preadipocyte and proliferation markers were more highly expressed in the stromal-vascular fraction of LWS than HWS chicks, greater expression of granzyme-A and the presence of more annexin V-positive cells suggests that apoptosis may limit the adipogenic potential of adipocyte precursor cells and represent a novel mechanism that regulates the expansion of adipose tissue. Results provide new insights on cellular mechanisms associated with adipose tissue development in the lean and obese state.

Key words: adipocytes, apoptosis, chicken, development, stromal-vascular fraction

Introduction

The Virginia lines of chickens differ more than 12-fold in body weight at 56 days of age after 60 generations of divergent selection for body weight at this age [1, 2]. Chickens from the high
body weight-selected (HWS) line are hyperphagic, obese and develop symptoms characteristic of metabolic syndrome, whereas the low body weight-selected (LWS) are hypophagic with some being anorexic, and all are lean [3]. The objective of this experiment was to separate the stromal-vascular fraction (SVF) and adipocyte compartments from adipose tissue of LWS and HWS chicks at day of hatch and day 14, and measure mRNA abundance of a variety of factors involved in different facets of adipogenesis and adipocyte function.

**Materials and methods**

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. Chicks were group-caged at 32 ± 1°C and 50 ± 5% humidity with 24-hour light conditions. On day of hatch and day 14 post-hatch, chicks with median body weights of their line were euthanized for tissue collection. Equal amounts of adipose tissue from 10 male chicks within each line were used to generate a pooled sample to ensure that there were enough cells to yield sufficient amounts of RNA. Four pools were generated for n = 4 experimental units per line within an adipose tissue depot and day.

Pooled samples were generated from the subcutaneous depot at day of hatch and the abdominal depot at day 14. At day of hatch, the LWS chicks do not have abdominal fat, whereas lines have similar amounts of subcutaneous adipose tissue [4], which is why subcutaneous samples were used for the day of hatch analysis. Pooled samples were minced into fine pieces and digested with 4-(2-hydroxyethyl) piperazine-1-ethanesulfonic acid (HEPES) solution (0.1 mol/L HEPES, 5 mmol/L d-glucose, 1.5% bovine serum albumin) containing 500 units/mL of Collagenase, Type I (Worthington Biochemical Corporation, NJ, USA), for 1 h at 37°C. Contents were passed
through 250-μm filters (Pierce, IL, USA) and filtrate was centrifuged at 186 × g for 10 min to separate adipocytes from the SVF. Adipocytes were washed 3 times with DMEM/F12 (Gibco, NY, USA) containing 10% defined fetal bovine serum (HyClone, UT, USA) and 1% penicillin/streptomycin (HyClone). SVF cell pellets were resuspended and incubated in red blood cell lysis buffer (155 mmol/L NH₄Cl, 10 mmol/L KHCO₃, 0.1 mmol/L EDTA), and then filtered through a 20-μm mesh (Celltrics, Germany). The filtrate was sequentially centrifuged at 1,000 × g for 5 min.

TRI Reagent ® (Molecular Research Center, OH, USA) was added to adipocytes and SVF pellets for cell lysis with a 21-gauge needle (BD, NJ, USA). Total RNA was isolated with the Direct-zol™ RNA MiniPrep kit (Zymo Research, CA, USA) according to the manufacturer’s instructions. Total RNA samples were quantified and integrity verified using RNA StdSens Analysis kits and an Experion Automated Electrophoresis System (Bio-Rad, CA, USA). Reverse transcription was performed with 200 ng of total RNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, NY, USA) as described [5, 6], except for the SVF from LWS at hatch, from which only 50 ng of total RNA was used due to the limited amount of RNA that could be obtained from the tissue. Primers were designed with Primer Express 3.0 (Applied Biosystems; Table 4.1). Real time PCR reactions were performed in 10 μL that contained 5 μL Fast SYBR Green Master Mix (Applied Biosystems), 0.25 μL each of 5 μM primers, and 3 μL of diluted cDNA (10× dilution from 200 ng and 2.5× from 50 ng samples). Real time PCR reactions were performed in duplicate as described [5, 6].

Data were analyzed using the ΔΔCT method, where ΔCT = CT target gene – CT actin, and ΔΔCT = ΔCT target sample – ΔCT calibrator [7]. The average of subcutaneous SVF and abdominal SVF samples from HWS were used as the calibrator samples for apoptosis-related
genes (granzyme A: GZMA, and caspase 3: CASP3) on day of hatch and day 14, respectively. The average of the ΔCT from HWS was used as the calibrator sample for all other genes on each sampling day. The fold difference (relative quantity: RQ) was calculated as $2^{-\Delta\Delta CT}$. Apoptosis-related gene RQ values were analyzed using the Fit Model platform, while all other data were analyzed by t-tests using the Fit Y by X Model platform of JMP Pro 13 (SAS Institute, NC, USA). The statistical model for GZMA and CASP3 RQ values included the main effects of genetic line and cellular compartment, and their interaction. Genetic line was the only effect in the model for other RQ data. Differences were considered significant at $P < 0.05$.

**Results**

Results for all genes except apoptotic markers are summarized in Table 4.2. At day of hatch, there was greater expression of adipocyte enhancer binding protein 1 (AEBP1) in the subcutaneous SVF of HWS than LWS. CD71 and Krüppel-like factor 1 (KLF1) mRNAs were greater in the subcutaneous SVF of LWS than HWS chicks.

In adipocytes at hatch, lipoprotein lipase (LPL) was greater in HWS than LWS, and diglyceride acyltransferase 2 (DGAT2) bordered on being more highly expressed in HWS. Sterol regulatory element-binding transcription factor 1 (SREBP1) tended to be greater in LWS than the HWS adipocytes at hatch. At day 14, CD71, delta like non-canonical notch ligand 1 (DLK1), GATA-binding protein 2 (GATA2) and KLF1 mRNAs were greater in LWS than HWS in the SVF of abdominal adipose tissue. In abdominal adipocytes at day 14, carnitine palmitoyltransferase 1A (CPT1A) mRNA was greater in LWS than HWS, whereas LPL and SREBP1 tended to be greater in HWS than LWS.
Results for *CASP3* and *GZMA* are summarized in Table 4.3. At hatch, HWS expressed more *GZMA* than LWS in the subcutaneous depot whereas at day 14, LWS expressed more than HWS in the abdominal depot. At day 14, *CASP3* was greater in the SVF than adipocytes, while *GZMA* mRNA was greater in adipocytes than the SVF.

**Discussion**

The LWS and HWS lines are predisposed to be lean and obese, respectively, and this is associated with differences in adipose tissue development during the early post-hatch period [4]. While the LWS accumulate little abdominal fat during the first two weeks post-hatch, the HWS line individuals become obese and must be food-restricted at sexual maturity in order to avoid developing metabolic syndrome [8]. Unexpectedly, there was greater gene expression of some adipogenic transcription factors in LWS than HWS [4], which prompted us to explore gene expression at the cellular level.

At day 14, there was greater expression of *CPT1A* in LWS than HWS. CPT1A catalyzes the rate-limiting step of fatty acid oxidation, and that this isoform is predominantly expressed in adipose tissue [9] is consistent with our observation that LWS have greater fatty acid oxidative activity and efficiency in abdominal fat than HWS [8]. Greater expression of *LPL* and *DGAT2* in HWS, on the other hand, likely contributes to their rapid accumulation of abdominal fat during the first two weeks post-hatch [4]. Differences in adiposity may also relate to numbers of cells with adipogenic activity and their proliferative or apoptotic potential. At hatch, there was greater expression of mesenchymal stem cell (MSC) marker *CD71* [10-12] and proliferation marker *KLF1* [13] in the SVF of subcutaneous fat in LWS than HWS, whereas HWS chicks expressed greater amounts of preadipocyte marker *AEBP1* [14, 15]. In abdominal fat at day 14, LWS
expressed more CD71, proliferation markers GATA2 and KLF1, as well as preadipocyte marker DLK1. These results suggest that viability of precursor cells limits adipose tissue expansion in LWS. We thus measured gene expression of apoptotic factors and observed that LWS had approximately 3 times greater expression of GZMA mRNA than HWS at day 14. GZMA induces apoptosis independently of CASP3 [16], suggesting that apoptosis might limit adipose tissue expansion in LWS, which was confirmed by flow cytometry. There were more annexin V-positive cells in the SVF of LWS than HWS chicks at day 14 (Figure 4.1). To date, there is no report of GZMA-mediated apoptosis in adipose tissue, or its relation to leanness or obesity in any species. This research provides novel insights into the different molecular mechanisms of adipocyte development and metabolism in the obese and lean states. Further research is required to understand the function of GZMA in the regulation of adipose tissue development.

Acknowledgements

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References


### Table 4.1. Primers used for real time PCR.

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<td>NM_205256.2</td>
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<td>NM_001012898.1</td>
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<td>ZNF423</td>
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1 Adipocyte enhancer binding protein 1: *AEBP1*; Adipose triglyceride lipase: *ATGL*; Caspase 3: *CASP3*; Transferrin receptor protein 1 / Cluster of differentiation 71: *CD71*; Carnitine palmitoyltransferase 1A: *CPT1A*; Diglyceride acyltransferase 2: *DGAT2*; Delta like
non-canonical notch ligand 1: DLK1; Fatty acid binding protein 4: FABP4; GATA-binding protein 2: GATA2; Granzyme-A: GZMA; Krüppel-like factor 1: KLF1; Lipoprotein lipase: LPL; Peroxisome proliferator-activated receptor gamma: PPARγ; Sterol regulatory element-binding transcription factor 1: SREBP1; Zinc finger protein 423: ZNF423.
Table 4.2. Adipose tissue cellular fraction mRNA at day of hatch and day 14 in chicks from anorexic and obese lines.

<table>
<thead>
<tr>
<th>Effect</th>
<th>SVF</th>
<th>Adipocyte</th>
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<tr>
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<td>CD71</td>
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<tr>
<td>DOH</td>
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<tr>
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<td>HWS</td>
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<tr>
<td></td>
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<tr>
<td>D14</td>
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<tr>
<td>LWS</td>
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<td>2.64</td>
</tr>
<tr>
<td>HWS</td>
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<td>SEM</td>
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</tr>
<tr>
<td></td>
<td>P-value</td>
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† Effect of genetic line (low body weight-selected line; LWS, high body weight-selected line; HWS) on mRNA in the stromal-vascular fraction (SVF) and adipocytes on day of hatch (DOH) in the subcutaneous fat depot and day 14 post-hatch (D14) in the abdominal fat depot. Values represent least squares means with pooled SEM and associated P-values (n = 4). Abbreviations: Adipocyte enhancer binding protein 1: AEBP1; Transferrin receptor protein 1 / Cluster of differentiation 71: CD71; Delta like non-canonical notch ligand 1: DLK1; GATA-binding protein 2: GATA2; Krüppel-like factor 1: KLF1; Zinc finger protein 423: ZNF423; Adipose triglyceride lipase: ATGL; Carnitine palmitoyltransferase 1A: CPT1A; Diglyceride acyltransferase 2: DGAT2; Fatty acid binding protein 4: FABP4; Lipoprotein lipase: LPL; Peroxisome proliferator-activated receptor gamma: PPARγ; Sterol regulatory element-binding transcription factor 1: SREBP1. *P < 0.05.
Table 4.3. Adipose tissue cellular compartment caspase 3 and granzyme-A mRNA.

<table>
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<tr>
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<td></td>
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<td></td>
</tr>
<tr>
<td></td>
<td>LWS</td>
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<td></td>
<td>HWS</td>
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<tr>
<td></td>
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<td></td>
<td>P-value</td>
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<td>0.42</td>
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<td>Compartment (C)</td>
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<td>SVF</td>
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<td>Adipocyte</td>
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<td></td>
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<table>
<thead>
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<th>Gene</th>
<th>Effects†</th>
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<tr>
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<td>Genetic line</td>
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<td>P-value</td>
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<tr>
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<td>SVF</td>
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<td>Adipocyte</td>
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<tr>
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<td>P-value</td>
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<tr>
<td></td>
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<td>0.84</td>
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</table>

†Effects of genetic line (L; LWS-low body weight-selected, HWS-high body weight-selected), compartment (C; stromal-vascular fraction: SVF, adipocyte), and their interaction (L × C) on caspase 3 (CASP3) and granzyme-A (GZMA) mRNA on day of hatch in the subcutaneous fat depot and day 14 post-hatch in the abdominal fat depot. Values represent least squares means with pooled SEM and associated P-values (n = 4). *P < 0.05.
Figure 4.1. Annexin V+ cell percentage in the stromal vascular fraction of abdominal adipose depot in chicks selected for low (LWS) or high (HWS) body weight at day 14 post-hatch. Cells were isolated and purified as described in the main text. The filtrate was then centrifuged at 1,000 × g for 5 min, followed by the removal of supernatant. The pellets were resuspended in 1× annexin binding buffer (Invitrogen, MA, USA), followed by the incubation with Alexa Fluor 594 conjugated annexin V (Invitrogen; 5 μL per 10⁵ cells) under room temperature for 15 minutes, after which the cells were washed and resuspended in annexin-binding buffer. DAPI (Invitrogen) was added at 1:10000 to the cell suspension to distinguish dead cells. Cell sorting was performed by BD FACS Aria Flow Cytometer (BD, NJ, USA). Percentages were compared by Student t-test. Data are presented as mean ± SEM. *P < 0.05.
Chapter 5: Early post-hatch stress induced DNA hypomethylation of corticotropin-releasing factor that disrupted binding of a methyl-CpG-binding domain protein in the hypothalamic paraventricular nucleus of chicks predisposed to anorexia

Abstract

When subjected to a combination of nutritional and thermal stressors at hatch, the low body weight-selected (LWS) line chicks, which are predisposed to anorexia, are refractory to the orexigenic effect of exogenous neuropeptide Y (NPY) and have increased expression of NPY in the arcuate nucleus (ARC) and corticotropin-releasing factor (CRF) in the paraventricular nucleus (PVN) than the non-stressed counterparts at day 5 post-hatch. We hypothesized that such changes are resulted from epigenetic modification. Our results showed that stress exposure reduced yolk resorption and resulted in lowered body weight, lean and fat masses in LWS chicks on day 5 post-hatch. Stress induced global hypermethylation and increased DNMT activity in the ARC but not PVN. There was no effect on the methylation status of CpG sites near the NPY gene. In the PVN of stressed LWS chicks, there was decreased methylation of a CpG site located at the core binding domain of methyl cytosine binding domain protein 2 (MBD2) in the CRF gene promoter, so as the binding of anti-MBD2 antibody to CRF promoter in the hypothalamus of stressed LWS. These results support that early post-hatch stress intensifies the anorexic condition in LWS chicks. There was reduced utilization of yolk reserves and impaired growth, and hypomethylation of a CpG site on the CRF promoter resulted in lower binding of the transcriptional repressor, MBD2. These findings provide novel insights on molecular mechanisms through which stressful events induce or intensify anorexia in predisposed individuals and a novel molecular target for further studies.
**Key words:** anorexia, ARC, chicks, CRF, DNA methylation, early-life stressor, hypothalamus, MBD2, NPY, PVN

**Introduction**

Appetite regulatory signals, stress responses, and thermoregulation are integrated at the level of the hypothalamus. Although environmental stressors can alter feeding behavior, the molecular mechanisms are unclear and effects that persist with age likely have an epigenetic underpinning. A model that can be used to study these mechanisms are the Virginia body weight lines of chickens. Selection for low or high juvenile body weight for more than 60 consecutive generations has resulted in correlated responses in appetite regulation, body composition, and stress susceptibility [1, 2]. One of the most striking differences in appetite regulation in the lines is with respect to the orexigenic effects of neuropeptide Y (NPY). Under some conditions, the LWS chicks are completely refractory to the food intake-stimulating effects of centrally administered NPY [3]. Specifically, this change in appetite regulation is stress-induced, as a combination of transient low temperature coupled to delayed access to food at hatch rendered the LWS but not HWS chicks resistant to the hunger-promoting effects of NPY. The LWS chicks hatched and reared under thermoneutral conditions with full access to food responded to NPY with increased food intake albeit not as robustly as HWS chicks, which responded vigorously under all conditions [4, 5]. NPY is one of the most potent orexigenic factors in mammals and birds [6], and these results suggest that increased anorexigenic tone overrides the effects of NPY, which is quite remarkable given the potency of NPY at stimulating food intake.

At the molecular level, there was increased mRNA expression of *NPY* in the arcuate nucleus (ARC) and corticotropin-releasing factor (*CRF*) in the paraventricular nucleus (PVN) of the
hypothalamus in stressed LWS but not HWS chicks at 5 days post-hatch [4]. The ARC is vital for appetite regulation and energy homeostasis [7]. Both orexigenic NPY and agouti-related peptide (AgRP) neurons and anorexigenic pro-opiomelanocortin and cocaine- and amphetamine-regulated transcript neurons are found in the ARC, which direct axons to second-order nuclei like the PVN [8]. The PVN suppresses appetite and it is the major source of CRF, which inhibits feeding behavior and is part of the stress response [9]. In addition to up-regulated CRF mRNA in the PVN, stressed LWS chicks also had increased circulating corticosterone [4, 5]. The reversal of resistance to NPY by injecting a CRF receptor antagonist prior to exposure to the stressors verified that CRF signaling in the PVN may override the orexigenic effect of NPY and exacerbate anorexia in LWS chicks [4].

Although the research presented here with the LWS line does not include changes and that persist post-sexual maturity, there is a growing body of evidence showing that stress at young ages can shape the development of an organism and lead to long-term physiological alterations, such as changing body composition and nutrient metabolism [10]. Thus, the organism is predisposed to chronic metabolic and mental disorders [11-14]. When an animal is stressed, CRF is secreted from the PVN to stimulate the release of adrenocorticotropic hormone (ACTH) from the anterior pituitary [15]. ACTH then acts on the adrenal cortex to regulate production of glucocorticoids, which provide negative feedback by suppressing CRF to maintain homeostasis [16]. However, exposure to stress early in life can alter the sensitivity of the hypothalamic–pituitary–adrenal axis, leading to phenotypes that are resilient or vulnerable to stress later in life [16].

Human studies suggest that epigenetic regulation of gene expression plays a critical role in the long-term effects of early-life exposure to stressors [13]. DNA methylation, catalyzed by DNA
methyltransferases (DNMTs) is one of the best understood epigenetic modification mechanisms [17]. The effects of early-life stress, such as famine [18, 19], infant-parental separation [20, 21] and cold exposure [11] on long-term physiological changes are associated with DNA methylation status. Both early-life stress [22] and adulthood chronic stress [23] altered CRF expression through DNA methylation. We hypothesized that stressor-induced changes in DNA methylation lead to increased hypothalamic expression of CRF and NPY in LWS. Thus, the objective herein was to determine the effects of stress on DNA methylation in the ARC and PVN of LWS chicks and to elucidate the associated molecular mechanism.

Materials and Methods

Animals and experimental design
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 National Research Council Publication, Guide for the Care and Use of Laboratory Animals. The LWS chicks were hatched at the Paul B. Siegel Poultry Research Center at Virginia Tech. Eggs were from age-contemporary breeders from the 61st generation. All experiments were done within one hatch. On the day of hatch, chicks were placed inside cardboard boxes (37 × 24 cm, n = 20 per box) and divided into stressed (S) and non-stressed (NS) groups. The boxes, experimental design, and stress protocol were the same as implemented in our previous stress studies [4, 5]. The chicks in the stress group were subjected to −20 °C for 6 minutes and then transferred to 22 °C for 24 hours without food or water provided. The control chicks were group caged at 32 ± 1 °C and 50 ± 5% relative humidity with free access to diet (21.5% crude protein and 3,000 kcal ME/kg) and water. On day 1 post-hatch (24 hours post-stress), all chicks were transferred to individual cages in which rearing conditions and food were the same as for the control group. Chicks had
visual and auditory contact with each other in the individual cages and were handled twice daily to adapt to handling. All chicks were sexed by gonadal inspection.

**Body composition**

Fat and lean masses were measured (n = 6 males per group) with a minispec LF90 NMR whole body composition analyzer (Bruker, MA, USA). The instrument was calibrated with a bottle of 500 g of canola seeds before use as recommended by the manufacturer. Each chick was scanned twice and the average of the duplicates was used for data analysis. Prior to the measurements, yolk sacs were completely removed from the euthanized chicks by excision through the navel and weighed. This procedure was necessary to eliminate the confounding effects of residual yolk nutrients on whole body composition. Sex was determined by visual inspection of the gonads.

**Nuclear protein extraction and DNMT activity**

On day 5 post-hatch, chicks were decapitated (head and neck) and then perfused via the carotid artery with hypotonic buffer (10 mM HEPES, pH 7.9, with 1.5 mM MgCl₂ and 10 mM KCl). Brains were mounted with Tissue-Plus O.C.T compound (Fisher HealthCare, TX, USA), snap-frozen in liquid nitrogen, and then sectioned in the direction from rostral to caudal in a cryostat at −10 °C into 500 μm thick coronal sections within 30 minutes post-perfusion. The PVN and ARC were collected at 7.4 and 5.4 interaural, respectively, based on the Kuenzel and Masson chicken stereotaxic atlas [24]. Nuclei were collected using sterile disposable biopsy punch instruments (1 mm; Braintrree Scientific Inc., MA, USA) and were immediately transferred to sterile microcentrifuge tubes containing 25 μL pre-mixed pre-extraction buffer provided in the EpiQuik Nuclear Extraction Kit I (Epigentek, NY, USA) and stored at -80 °C until further processing. To ensure anatomical accuracy, the remaining brain section was photographed and anatomy confirmed via digital overlays containing the respective nucleus boundaries according to the
Kuenzel and Masson chicken stereotaxic atlas [24]. The nuclear extraction procedure was performed with the EpiQuik Nuclear Extraction Kit I (Epigentek), following the manufacturer’s protocol. Protein concentration was determined with a Bradford assay (Bio-Rad, CA, USA). The EpiQuik DNMT Activity/Inhibition Assay Ultra Kit-Colorimetric (Epigentek) was used to determine DNMT activity, following the manufacturer’s protocol with 10 μg of each of the nuclear extracts. The absorbance (optical density; OD) was detected at 450 nm using a M200 Pro Multi-Mode plate reader (Tecan, Switzerland). Specific activity was calculated as:

\[
\text{DNMT activity (OD/h/mg)} = \frac{\text{Sample OD} - \text{Blank OD}}{10 \, \mu g \, \text{Protein input} \times 2} \times 1000
\]

Six to nine samples from each treatment group were used for statistical analysis after exclusion of those that exceeded a two-fold standard deviation (SD) from the average.

**Genomic DNA extraction**

On day 5 post-hatch, chicks were decapitated (head and neck) and then perfused via the carotid artery with 1.5 mL of nucleic acid stabilizing buffer (16.7 mmol/L sodium citrate, 13.3 mmol/L EDTA and 3.5 mol/L (NH₄)₂SO₄; pH 5.2). Brains were sectioned and the PVN and ARC were collected as described above. Collected nucleus samples were immediately transferred to sterile microcentrifuge tubes containing DNA lysis buffer (Norgen Biotek, ON, Canada). Samples were vortexed, snap-frozen in liquid nitrogen, and stored at −80 °C until further processing. The genomic DNA was isolated according to the manufacturer’s instructions for the Cells and Tissue DNA Isolation Micro Kit (Norgen Biotek). The concentration and purity of total DNA was assessed with a Nanophotometer Pearl spectrophotometer (Implen, CA, USA) at 260/280/230 nm. Genomic DNA quality was verified by 1.2% agarose gel electrophoresis. Six to 7 genomic DNA samples from each group were used for global DNA methylation
quantification and 6 to 8 genomic DNA samples from each group were used for bisulfite conversion and downstream sequencing after exclusion of those that exceeded a two-fold SD from the average.

**Global DNA methylation quantification**

Global DNA methylation was quantified with a MethylFlash Methylated DNA Quantification Kit-Colorimetric (Epigentek) following the manufacturer’s protocol. The input DNA amount was 100 ng per reaction. The absorbance was measured at 450 nm and the percentage of DNA methylation (5-methyl cytosine %; 5-mC %) was calculated according to the following:

\[
5 - \text{mC} \% = \frac{(\text{Sample OD} - \text{Negative control OD}) \div 100 \text{ ng DNA input}}{(\text{Positive control OD} - \text{Negative control OD}) \times 2 \div 5 \text{ ng Positive control}}
\]

**Bisulfite conversion, PCR, and sub-cloning**

Bisulfite conversion was performed with the Epitect Fast DNA Bisulfite Conversion Kit (Qiagen, Germantown, MD) following the manufacturer’s instructions, with 100 ng of genomic DNA per reaction. Specific CpG-rich fragments in the promoter regions of CRF and NPY were amplified with EpiTaq HS (TaKaRa Bio Inc., Japan). The primers were designed with MethPrimer [25] (Table 5.1). Each 50 μL PCR reaction contained 100 ng bisulfite-converted DNA, 1.25 U EpiTaq HS, 5 μL Mg2+-free 10x PCR buffer, 2.5 mM MgCl2, 0.3 mM dNTP, 0.4 μM each of sense and anti-sense primers, and nuclease-free water. PCR was performed with a Bio-Rad T100 Thermocycler (Bio-Rad) under the following conditions: initial denaturing step at 95 °C for 30 seconds followed by 40 cycles of 1) 95 °C for 20 seconds, 2) 51-61 °C (51 °C for CRF primers amplifying -288 ~ 35 bp upstream of the transcription start site (TSS), 61 °C for CRF primers amplifying -805 ~ -645 upstream of TSS, and 52 °C for both sets of NPY primers) for 30 seconds, and 3) 72 °C for 30 seconds, and the final extension at 72 °C for 5 minutes. PCR
products were resolved on 1.2% agarose gels from which the bands were excised and purified with a MinElute Gel Extraction Kit (Qiagen), following the manufacturer’s protocol. Purified PCR products were ligated into the pGEM-T easy vector system (Promega, WI, USA) at 3:1 or higher insert/vector ratio and incubated at 4 °C overnight. Ligated vectors were then transformed into JM109 competent cells (Promega) following the manufacturer’s protocol (including positive and negative controls) and plated onto LB plates with 100 μg/mL ampicillin (Sigma, MO, USA), 0.5 mM isopropylthio-β-galactoside (IPTG; Sigma), and 80 μg/mL 5-bromo-4-chloro-3-indolyl-β-d-galactopyranoside (X-Gal; Promega), and cultured at 37 °C overnight for blue-white screening. From each sample, 6 white colonies were amplified at 37 °C, 225 × rpm, overnight, and plasmid DNA was extracted using a QIAprep Spin Miniprep Kit (Qiagen), following the manufacturer’s instructions. The concentration and purification of DNA were determined as described above. Prior to sequencing, target product sizes were verified for each sample by 1.2% agarose gel electrophoresis after EcoRI digestion.

**DNA sequencing and sequence analysis**

Purified plasmid DNA samples were submitted to the Bioinformatics Institute at Virginia Tech for Sanger Sequencing. Sequences were viewed in 4Peaks (Nucleobyes, the Netherlands) and analyzed by Bisulfite Sequencing DNA Methylation Analysis (BISMA) software online. The analysis parameters were set as default with a lower threshold conversion rate of 95%, lower threshold sequence identity of 90%, upper threshold of N-sites at cytosine positions and upper threshold gaps of 20% allowed, and the detection of clonal molecules and alignment as suggested by the software. The methylation frequency of a single CpG site in each sample was determined from the 6 sequenced clones as the percentage of the 6 clones in which the site was methylated. The average of percent methylation was then determined for the group and the
average used for statistical analysis. Overall methylation rate of a sample was calculated as
(methylated CpG sites / all CpG sites in the amplicon) × 100 %.

**Chromatin immunoprecipitation (ChIP) assay**

On day 5 post-hatch, chicks were decapitated (head and neck) and the whole hypothalamus were collected as described in [5] except that the hypothalamus samples were directly snap-frozen in the liquid nitrogen instead of being submerged in RNA stabilizing buffer. Samples were quickly minced into small pieces on ice and cross-linked in 1% formaldehyde solution for 5 minutes, which was then halted with 0.125 M glycine. Tissues were further homogenized with a Kinematica Polytron PT 10/35 GT Homogenizer (Kinematica Inc., NY, USA) for 30 seconds and washed twice with ice-cold 1× phosphate-buffered saline (PBS; Hyclone, UT, USA), followed by a 10-minute incubation on ice in cell lysis buffer [10 mM Tris-HCl (pH 8.0), 10 mM NaCl with 0.2% nonidet P-40 and 1× protease inhibitor cocktail (Thermo Scientific, IL, USA)]. Samples were then incubated in assay buffer (1% nondiet P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate and 0.004% sodium azide in 1× PBS) on ice for 10 minutes, followed by sonication at 30 seconds on / 30 seconds off for 30 cycles using a Bioruptor 300 with circulating chilled water system (Diagenode, Inc., NJ, USA). Forty-nine μL of sheared chromatin together with 1 μg of anti-methyl binding domain protein 2 (MBD2) antibody (Sigma) were used for each ChIP reaction using ChromaFlash One-Step ChIP kit (Epigentek) according to the manufacturer’s instructions. Two microliters of reverse-crosslinked DNA were added to each 20 μL PCR reaction containing 10 μL Fast SYBR Green Master Mix (Applied Biosystems, CA, USA), 1 μL of 0.5 μM forward (5’-GGAGGCAGATTGCATACAGGA-3’) and reverse (5’-CCTCACAGAAGGCCCTAC-3’) strand primers and 6 μL nuclease-free water. The primers were designed with Primer Express, version 3.0 (Applied Biosystems) which amplifies CRF at -
815 ~ -631 bp upstream of the TSS. Reactions were performed under the conditions: 95 °C for 7 min followed by 40 cycles of 95 °C for 10 seconds and 60 °C for 30 seconds, and the final extension was at 72 °C for 1 min. Relative fold enrichment (FE) was calculated as

\[ FE = 2^{(Ct_{IgG} - Ct_{Sample})}, \]

where IgG was used as the negative control in the ChIP assay.

**Statistical analysis**

Body composition, DNMT activity, global DNA methylation and ChIP data were analyzed by \(t\)-tests and sequence data were analyzed by the Wilcoxon test using the Fit Y by X Model of JMP Pro 14 (SAS Institute, NC, USA). Because the stressed LWS had no fat in contrast to non-stressed ones, these data were analyzed by Pearson’s chi-square test in JMP Pro 14. Because preliminary statistical analysis with 2-3 replicates in each sex group and previous studies \[4, 5\] showed no sex effect, treatment (non-stressed [NS] vs. stressed [S]) was the only effect included in the analyses. Differences were considered significant at \(P < 0.05\). For all experiments, sample size information is provided in figures and reflects numbers after exclusion of outliers (greater than ± two standard deviations of the mean).

**Results**

**Body weight, body composition and yolk sac weight**

Stressor-exposure was associated with reduced body weight (\(P = 0.008\); **Figure 5.1A**) and lean mass (\(P = 0.007\); **Figure 5.1B**) in LWS chicks at day 5 post-hatch. Stressor-exposed chicks had more yolk sac remaining than their non-stressed counterparts, both as an absolute weight (\(P = 0.02\); **Figure 5.1C**) and as a percentage of body weight (\(P = 0.02\); **Figure 5.1D**). Fat mass was not detectable in the stressed chicks at 5 days post-hatch (data not shown), whereas the control
birds had an average of $0.72 \pm 0.15$ g of fat, accounting for $2.78 \pm 0.56\%$ of their body weight. Thus, fat mass was different by stress status ($\chi^2(1) = 6, P = 0.01$).

**DNMT activity and global DNA methylation**

In the ARC, DNMT activity was increased in stressed chicks at 5 days post-hatch ($P = 0.04$; **Figure 5.2**), whereas in the PVN, there was no difference in DNMT activity between the two groups. The same pattern was observed for global DNA methylation (**Figure 5.3**), where 5-methyl cytosine (5-mC) in the ARC was greater in stressed than non-stressed control chicks ($P = 0.01$), with no difference between groups in the PVN.

**CpG site methylation at the CRF and NPY genes**

We measured the methylation frequency of individual CpG sites near the *CRF* (**Figure 5.4**) and *NPY* (**Figure 5.5**) genes. One out of 31 CpG sites that were assessed in *CRF* differed (**Figure 5.4C-D**), where methylation was decreased in response to the stressors at 5 days post-hatch ($P = 0.04$). The overall methylation of all CpG sites in *CRF* and *NPY* was similar between groups (**Figure 5.4A-B and 5.5A-B**, respectively), as was the methylation frequency of all 61 individual CpG sites that were sequenced on the *NPY* gene (**Figure 5.5C-D**). The overall methylation rate of CpG sites 4-20 from -96 upstream to 416 bp downstream of the TSS of the NPY gene was approaching significance ($P = 0.0811$), where methylation in stressed chicks tended to be lower than the non-stressed ones (**Figure 5.5E**).

**Binding of MBD2 to the promoter region of CRF**

Relative fold enrichment of DNA reverse crosslinked from anti-MBD2 antibody-bound chromatin is shown in **Figure 5.6**. DNA enrichment was lower in the stressed than the non-stressed chicks ($P = 0.02$).
Discussion

The hypothalamus has a critical role in integrating appetite and stress regulatory responses in order to maintain metabolic homeostasis. The LWS chicks that are exposed to a combination of nutritional and thermal stressors upon hatching are resistant to the orexigenic effects of centrally administered NPY 5 days later [5] and overriding anorexigenic tone originating from CRF production in the PVN plays an important role in the magnification of anorexia in these chicks [4]. We thus hypothesized that there was accelerated depletion of the yolk sac, the primary energy source for newly hatched chicks [26], upon exposure to stressors. However, at day 5 post-hatch, stressed chicks had greater amounts of yolk remaining (both on an absolute and relative to body weight basis) coupled to lower body weights than their non-stressed counterparts.

A similar phenomenon was observed in obesity-prone broiler chicks that were deprived of food immediately after hatching; they had more yolk remaining during the first 3 days post-hatch, and gained less weight [27]. In another study, chicks were housed in a cold environment (5 °C lower than optimal temperature) for 5 days with ad libitum or delayed access to food for 24 hours right after hatch. Cold-exposed chicks showed no difference in growth from their control counterparts, whereas daily food intake and yolk resorption rates were greater, demonstrating that more energy is required to generate heat to maintain thermoneutrality. The chicks with 24-hour food deprivation weighed less than the controls from day 2 to 5 post-hatch. Although there was more potent yolk resorption immediately after the 24-hour food deprivation, the remaining yolk mass was greater than in the controls at the end of the 5-day trial. Without exogenous food, yolk is the only source of energy for survival, and its nutritional constituents are utilized efficiently through endocytosis (into the bloodstream). In the presence of food, however; more of the yolk is resorbed through the small intestine via the yolk stalk, demonstrating the importance of early-life
nutrition to support optimal development of the gastrointestinal tract, where the presence of nutrients is an important stimulus for cellular growth [28]. In the present study, the combination of transient exposure to cold temperature and food deprivation likely induced a change in metabolism distinct from the effects of either stressor alone. This may explain why changes induced by thermal stress or nutritional deprivation in other studies produce distinct effects on appetite regulation, growth, and nutrient utilization.

Because the LWS chicks were exposed to cold with no access to food during the first 24 hours post-hatch, and yolk utilization was not increased, we determined if mobilization of body reserves occurred to fulfill energy requirements for survival. As expected, stressed chicks had less lean mass and negligible amounts of fat. Exposure to a colder ambient temperature for 3 weeks post-hatch reduced broiler chick body weight gain, however body composition was not determined in that study [29]. In diet-induced obese mice, adulthood chronic stress decreased body fat mass and increased lean mass (both on a percent body weight basis) [30]. Although the effects of exposure to stressors at early ages on the alteration of nutrient metabolism during adulthood has been reported in rats [10], macaques [31] and mice [32], the effect on early neonatal body composition is unclear, especially in individuals predisposed to anorexia. To our knowledge, this is the first demonstration of effects of stress on early-life changes in body composition. Results herein indicate that increased mobilization of body reserves, instead of accelerated yolk resorption, may be the primary means of survival of stressor-exposed LWS chicks, which are inherently hypophagic and predisposed to anorexia.

In our previous studies, there was up-regulation of CRF in the PVN and NPY in the ARC of stressor-exposed LWS chicks, and we postulated that CRF may override the orexigenic effect of NPY upon exposure to stressors and exacerbate anorexia in the LWS chicks at a later age [4, 5].
In the present study, we determined whether such changes are related to stressor-induced alterations in DNA methylation patterns. There are more than 20,000 CpG islands in the chicken genome, and the global methylation rate of these islands is below 10% on average in different tissues of red jungle fowl and broilers [33]. This is in line with our observation that the amount of global DNA methylation in the ARC and PVN was below 1% regardless of treatment group. The exposure to stressors was associated with an increase in global methylation and DNMT activity in the ARC, but not the PVN. The assay for measuring DNMT activity does not distinguish among enzyme sub-types (de novo DNMT 3A and 3B vs. maintenance DNMT1). As global DNA methylation in a nucleus likely does not reflect the methylation changes at individual loci, we determined the DNA methylation patterns in CpG islands identified in the promoter region of NPY and CRF.

Although the critical role of CRF in mediating stress responses has been comprehensively reviewed [34], little is known regarding the mechanism through which CRF is upregulated by stressors. Females being stressed during early pregnancy resulted in de-methylation at specific CpG sites in the CRF promoter region in the hypothalamus and amygdala of the offspring, and this was associated with increased CRF expression in the amygdala [35]. Here we show that upon stressor exposure, there is reduced methylation status of a CpG site near the CRF promoter, in the PVN, that corresponds to the putative MBD2 binding domain. MBD2 is one of five methyl binding domain proteins found in mammals with each MBD protein binding to its preferred sequences [36]. It shows the greatest selectivity of methylated against unmethylated CpG sites, which facilitates more rapid and stable binding to methylated CpGs [37]. MBD2 is also found in chickens, sharing 83% sequence identity with human MBD2 [38]. The core binding sequence of chicken MBD2 is T(mC)GG [39], the same as the sequence containing the differentially-
methylated CpG in *CRF* in the PVN of stressed LWS chicks. DNA binding to MBD2 stabilizes the structure of the latter, which facilitates recruitment of the nucleosome remodeling and deacetylase co-repressor complex to repress transcription [40]. Thus, heavy methylation (~80%) of this CpG site in control chicks is likely associated with greater binding to the MBD2 protein, thereby permitting the recruitment of repressors to silence *CRF* expression under the non-stressed state. When LWS chicks were stressed immediately post-hatch, the methylation state of this CpG site was reduced substantially at day 5 post-hatch. We speculate that this is associated with reduced MBD2 recruitment, which results in an environment that is more permissible to transcriptional activation of *CRF* in order to modulate the downstream stress responses. This speculation is supported by our previous observations that *CRF* mRNA is up-regulated in the PVN of stressor-exposed LWS [4], which is coupled to increased circulating corticosterone [5]. The ChIP results further confirmed this speculation that binding of MBD2 in the promoter region of *CRF* was disrupted in the stressed chicks as a consequence of reduced methylation at a CpG site in its binding domain. That other CpG sites in *CRF* that we sequenced were all lowly methylated is consistent with the observation that methylation in the chicken *CRF* promoter region is low, and distinct regions of the *CRF* gene respond differently to stressors [16]. For instance, DNA methylation is enriched in the gene body, whereas gene expression is negatively correlated with methylation in the promoter region [33].

We also determined the methylation status of the *NPY* gene and our analysis covered 60 out of 78 CpG sites, close to the transcription start site (TSS) of *NPY* (both downstream and upstream), where there is high sequence conservation with the mammalian *NPY* genes and enrichment of predicted bindings sites for transcription factors [41]. Though not reaching statistical significance, the overall methylation rate of CpG sites at -46 upstream to 70 bp downstream of
the TSS in \( NPY \) tended to be lower in the stressed chicks than the non-stressed ones. However, no methylation difference was detected for individual CpG sites sequenced in the \( NPY \) promoter region. Therefore, it is unclear if increased mRNA expression of \( NPY \) was related to changes in methylation within this region. In addition to its potent orexigenic effect, NPY also shows anti-stress effects in response to a variety of stressors (reviewed in [42]). Upon stressor exposure, CRF regulates the production of glucocorticoids, which are capable of upregulating the expression of NPY to alleviate the stress response and restore homeostasis [43]. Thus, upregulated \( NPY \) expression may not be directly modulated by methylation changes upon stressor exposure, but it could be a downstream target in the stress response. It is also possible that expression is regulated through other mechanisms, such as histone modifications or binding of transcriptional activators, or that other methylation sites not evaluated in this study were affected. Because we did not detect changes in methylation at the \( NPY \) gene nor evaluate other genes in the ARC, it is unclear whether the global hypermethylation observed in the ARC is related to changes in genes that inhibit appetite or induce satiety.

**Conclusions**

Early post-hatch exposure to a combination of thermal and nutritional stress altered the utilization of yolk and impaired growth of the low body weight-selected chicks that are all hypophagic and predisposed to anorexia. Early post-hatch stress was associated with increased DNMT activity and global hypermethylation in the ARC but no change in the PVN. Results from gene-specific methylation analyses suggest that upregulation of \( CRF \) expression in the PVN of stressor-exposed chicks is associated with hypomethylation of a MBD2 binding site. Consequently, disrupted binding of transcriptional repressor MBD2 is likely associated with increased transcription of \( CRF \). NPY may be a downstream effector of the stress response that is
not directly modulated by DNA methylation. Our study provided further implications for understanding the molecular basis for eating disorders and identifying novel strategies to alleviated stress-induced changes in eating behavior.

Acknowledgements

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Conflict of Interest

The authors have no conflict of interest to report.
References

10. Maniam, J., et al., Early life stress induced by limited nesting material produces metabolic resilience in response to a high-fat and high-sugar diet in male rats. Front Endocrinol (Lausanne), 2015. 6(138).
32. Yam, K.Y., et al., *Exposure to chronic early-life stress lastingly alters the adipose tissue, the leptin system and changes the vulnerability to western-style diet later in life in mice.* Psychoneuroendocrinology, 2017. **77**: p. 186-95.


Table 5.1. Primers for Bisulfite PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequence (5'-3'); Forward/Reverse</th>
<th>Genomic location</th>
<th>Amplicon length (bp)</th>
<th>Number of CpG sites</th>
<th>Location (upstream of TSS)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>CRF</strong></td>
<td>AATCTCATTCAATATTTTTA/ GAATTGTGATTAGATTTGG</td>
<td>chr2: 115012110-115012433</td>
<td>324</td>
<td>24</td>
<td>-288 ~ 35</td>
</tr>
<tr>
<td></td>
<td>GGATGTGTAATTTGAAGGAGGTAGA/ AACAAATCCCTCTAAAAATCCCTTTA</td>
<td>chr2:115012790-115012952</td>
<td>161</td>
<td>7</td>
<td>-830 ~ -670</td>
</tr>
<tr>
<td><strong>NPY</strong></td>
<td>AAAACACCATAAAACTATAA/ TTAGAGAAGGAGTAGTTTAG</td>
<td>chr2: 31464313-31464554</td>
<td>242</td>
<td>16</td>
<td>175 ~ 416</td>
</tr>
<tr>
<td></td>
<td>ATAGTTTTTTAGAAGGTAAGTTATGGG/ AATATCAAATCAATACCACAAAACTC</td>
<td>chr2: 31463545-31463709</td>
<td>165</td>
<td>7</td>
<td>-594 ~ -430</td>
</tr>
<tr>
<td></td>
<td>GTTTAAGGTTTTTTTGTGTTGT/ ACCTCATAATACCCCTACATCTAAAC</td>
<td>chr2: 31464042-31464328</td>
<td>287</td>
<td>38</td>
<td>-96 ~ 190</td>
</tr>
</tbody>
</table>

\(^1\)Chicken corticotropin-releasing factor (CRF) and neuropeptide Y (NPY) gene sequences were analyzed with MethPrimer and UCSC genome browser (https://genome.ucsc.edu/) to identify CpG islands in proximity to the promoter region. The location relative to the transcription start site (TSS) is indicated above.
Figure 5.1. Body weight (A), Lean mass (B), Yolk sac weight (C), and Yolk sac weight (YSW) on a body weight (BW) basis (D) of non-stressed (NS) and stressed (S) LWS chicks on day 5 post-hatch. Data were analyzed by $t$-tests and presented as means ± SEM. *$P < 0.05$, n = 6 per group.
Figure 5.2. DNA methyltransferase (DNMT) activity in the arcuate nucleus (ARC) and paraventricular nucleus (PVN) of non-stressed (NS) and stressed (S) LWS chicks on day 5 post-hatch. Data (n = 7 for ARC NS, n = 6 for ARC S and n = 9 for PVN) were analyzed by t-tests and presented as means ± SEM. *P < 0.05.
Figure 5.3. Global DNA methylation (5-methyl cytosine percentage; 5-mC%) in the arcuate nucleus (ARC) and paraventricular nucleus (PVN) of non-stressed (NS) and stressed (S) LWS chicks on day 5 post-hatch. Data (n = 6 for ARC NS and PVN S groups and n = 7 for ARC S and PVN NS groups) were analyzed by t-tests and presented as means ± SEM. *P < 0.05.
Figure 5.4. CpG site methylation status at the CRF gene in the paraventricular nucleus (PVN) of non-stressed (NS) and stressed (S) LWS chicks on day 5 post-hatch. (A-B) Overall methylation frequency of 7 CpG sites within -830 ~ -670 bp upstream of the transcription start site (TSS) (5’→3’; A) and 24 CpG sites from upstream -288 to downstream 35 bp of the TSS (5’→3’; B) of the CRF gene; (C) Methylation rate of each CpG site within -830 ~ -670 bp upstream of the TSS; (D) Methylation rate of each CpG site from upstream -288 to downstream 35 bp of the TSS (omitted sites in which no methylation was detected). Methylation rates in NS and S chicks (n = 7 for NS and n = 8 for S except for Figure 4C, where n = 6 for NS and n = 7 for S) were analyzed by the Wilcoxon test and presented as means ± SEM. *P < 0.05.
Figure 5.5. CpG site methylation status at the NPY gene in the arcuate nucleus (ARC) of non-stressed (NS) and stressed (S) LWS chicks on day 5 post-hatch. (A-B) Overall methylation frequency of 7 CpG sites within -594 ~ -430 bp upstream of the transcription start site (TSS) (5’→3’; A) and 54 CpG sites from upstream -96 to downstream 416 bp of the TSS (5’→3’; B) of the NPY gene; (C) Methylation rate of each CpG site within -594 ~ -430 bp upstream of the TSS; (D) Methylation rate of each CpG site from -96 upstream to 416 bp downstream of the TSS (omitted sites in which there was no methylation); (E) Methylation rate of CpG sites 4-20 at -96 upstream to 416 bp downstream of the TSS. Methylation rates in NS and S chicks (n = 8) were analyzed by the Wilcoxon test and presented as means ± SEM.
Figure 5.6. Relative fold enrichment of DNA recovered from anti-MBD2 antibody-bound chromatin in the hypothalamus of non-stressed (NS) and stressed (S) LWS chicks on day 5 post-hatch. Data (n = 10 for NS and n = 12 for S) were analyzed by t-tests and presented as means ± SEM. *P < 0.05.
Chapter 6: Short-term fasting and refeeding induced changes in adipose tissue physiology in 7-day old Japanese quail

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Abstract

Adipose tissue development is influenced by a variety of factors, including nutrition and genetic background. Among avian species, the most is known in chickens and it is unclear if other less-artificially-selected birds are similar during the first week post-hatch. The aim of this study was thus to determine effects of fasting and refeeding on adipose tissue physiology in Japanese quail (Coturnix japonica). On day 7 post-hatch, quail were randomly assigned to fed (control), 6 hours of fasting (fasted), or 6 hours of fasting followed by 1 hour of refeeding (re-fed) groups. Blood samples were collected for plasma non-esterified fatty acid (NEFA) determination and subcutaneous adipose tissues were harvested for gene expression analyses. Plasma NEFAs were elevated in the fasted state and restored to baseline within 1 hour of refeeding. In the adipose tissue, CCAAT/enhancer binding protein α mRNA was decreased by fasting and this change persisted through refeeding, whereas neuropeptide Y receptor 5 (NPY5R) mRNA was decreased in re-fed compared to fasted birds. Our results suggest that fasting promotes lipolysis and gene expression changes in young quail with some of these changes restored to original levels within only 1 hour of refeeding. Thus, in quail, adipose tissue physiology is dynamic and influenced by short-term changes in nutritional status during the early post-hatch period.

Key words: Japanese quail, fasting, refeeding, adipose tissue, non-esterified fatty acids
Introduction

Fasting is encountered in a variety of situations across species, yet short-term effects of fasting and refeeding on adipose tissue metabolism in young animals, especially avian species, are unclear. Three hours of fasting increased plasma non-esterified fatty acid (NEFA) concentrations in 4-day-old broiler (meat-type) chicks fed high-carbohydrate or high-fat diets, and altered mRNA abundance of lipid metabolism-associated factors in multiple adipose tissue depots [1]. In 7-day-old Japanese quail (Coturnix japonica), 3 and 6 hours of fasting followed by 1 hour of refeeding induced changes in appetite regulation, specifically hypothalamic mRNA abundance, with the effects more pronounced at 6 than 3 hours [2]. Effects of fasting on adipose tissue physiology in young quail are unknown, thus the aim of this study was thus to determine adipose tissue metabolism changes in 7-day-old Japanese quail in response to 6 hours of fasting and 1 hour of refeeding.

Materials and methods

Animals and experimental procedure

All 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. Japanese quail were bred and hatched at Virginia Tech as described [3]. Quail were group-caged in a brooder for 4 days post-hatch and then transferred to individual wire cages (8 cm width × 7 cm depth × 8 cm height) in a room maintained at a constant temperature of 35 ± 1 °C and 50 ± 5% relative humidity with a 14 hour light/10 hour dark period (lights on at 05:00 h). The individual cages allowed visual and auditory contact with other quail. To minimize the potential effects of handling stress on data collection, quail were
handled twice a day after being transferred into the individual cages for adaptation. The detailed handling procedure can be found in [2], and was demonstrated to have no influence on plasma corticosterone concentrations [3]. Prior to the beginning of the experiment, quail were provided free access to tap water and a corn-soybean meal-based mash starter diet (2900 kcal ME/kg and 24% CP) formulated to meet the requirements of quail [4]. At 05:00 h on day 7 post-hatch, each quail was randomly assigned to one of the 3 feeding groups—fed group, which was fed ad libitum throughout the experiment; 6 hour fasting group, which was deprived of food from 05:00 h to 11:00 h; and 6 hours of fasting with 1 hour of refeeding group, which after 6 hours of food deprivation was given free access to food for 1 hour (11:00 h to 12:00 h). All quail had free access to water during the experiment (05:00 to 12:00 h). Plasma (n = 10) and subcutaneous adipose tissue (n = 8-12) samples were collected from all groups. Quail were sex-sorted after gonadal inspection.

Plasma non-esterified fatty acids (NEFAs)

The procedure for plasma NEFA determination was the same as described in our previous studies [5, 6]. Briefly, blood was collected from the trunk of each quail via Microvette capillary blood collection tubes (Sarstedt, Germany) immediately following euthanasia and decapitation. After collection, samples were centrifuged at 2,000 g at room temperature to isolate plasma, which was then placed on ice and aliquoted. Plasma NEFA concentrations were measured with a NEFA-HR2 kit (FUJIFILM, Wako Diagnostics, Mountain View, CA) according to the manufacturer’s instructions. Absorbance was measured at 550 nm using an Infinite M200 Pro multi-mode plate reader (Tecan, Männedorf, Switzerland). 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**

Subcutaneous adipose tissue samples (all exposed adipose tissue under the skin above the cloaca after peeling back the skin) were collected and submerged in RNAlater (Qiagen, Valencia, CA). Tissues were homogenized in 1 ml Tri-Reagent (TR 118; Molecular Research Center, Cincinnati, OH) using 5-mm stainless steel beads (Qiagen) and a Tissue Lyser II (Qiagen) for 2 × 2 min at 25 Hz. The manufacturer’s instructions were followed to separate total RNA, and, after the step of addition to 100% molecular biology-grade ethanol, the RNA and ethanol mixtures were transferred to spin columns and further purified with the optional RNase-free DNase I from Direct-zol RNA kits (Zymo Research, Irvine, CA). A high-capacity cDNA Reverse Transcription Kit (Applied Biosystems, Carlsbad, CA) was used to synthesize single-stranded cDNA, following the manufacturer’s instructions. Primers for real-time PCR were designed with Primer Express 3.0 software (Applied Biosystems) and validated to have similar (within 5% of reference gene) amplification efficiency before use (Table 6.1). Real-time PCR was performed as described in [7].

**Statistical analysis**

All data were analyzed using the Fit Model procedure of JMP Pro 14 (SAS Institute, NC, USA). The real-time PCR data calculations employed the ΔΔCT method, where ΔCT = CT target gene − CT actin, and ΔΔCT = ΔCT target sample − ΔCT calibrator [8]. The average of the fed group was used as the calibrator. The fold difference (relative quantity) was calculated as $2^{-\Delta\Delta CT}$.

Effects involving sex were not significant; thus sex was excluded from the statistical model. The statistical model thus only included the main effect of treatment. Tukey’s test was used post hoc to separate the means. All data are presented as least squares means ± SEM. Differences were considered significant at $P < 0.05$. 
Results

Body weights did not differ among quail that were fed ad libitum (19.79 ± 0.60 g), fasted for 6 hours (19.64 ± 0.60 g), or fasted and then refed for 1 hour (19.59 ± 0.60) (Figure 6.1). Plasma NEFA concentrations were elevated in fasted compared to fed ($P < 0.0001$) and refed ($P < 0.0001$) quail (Figure 6.2). mRNA abundance in subcutaneous adipose tissue are shown in Table 6.2. Fasting and/or refeeding altered CCAAT/enhancer binding protein $\alpha$ ($C/EBP\alpha$; $P = 0.0002$) and neuropeptide Y receptor 5 ($NPY5R$; $P = 0.02$) mRNA. The expression of $C/EBP\alpha$ was greater in fed than fasted or refed quail. In contrast, $NPY5R$ mRNA was greater in fasted than refed quail, while values in fed quail were intermediate and not different from either of the two other groups.

Discussion

The aim of this study was to determine the effect of fasting and refeeding on adipose tissue physiology in 7-day-old Japanese quail. Six hours of fasting was not sufficient to alter body weight, a result consistent with those observed in adult Japanese quail that were subjected to 12 hours of fasting [9]. Prolonged fasting (from 24-hour to 21-day) reduced body weights [9-11]. Plasma NEFAs are considered to be an indirect indicator of adipose tissue lipolysis, as concentrations of free fatty acids in the blood almost exclusively originate from adipose tissue lipolysis [12]. The increased plasma NEFAs in 6-hour fasted quail resembles adult quail that were fasted for both short (4 hours) [13] and long (1 to 4 days) [11] periods of time, as well as 4-day-old broilers that were fasted for 3 hours after consuming high-carbohydrate or high-fat diets [1]. These results suggest that short-term fasting is sufficient to induce lipolysis and is sustained during longer periods of food withdrawal. Meanwhile, the restoration of plasma NEFAs to
baseline within 1 hour of refeeding after fasting suggests rapid recovery from fasting-induced lipolysis in quail chicks, consistent with 21-day old broilers and quail, where increased lipolytic activity from 24 hours of fasting was restored to fed control levels after 4 hours of refeeding [14]. In mice, fasting preferentially mobilizes adipose tissue from visceral depots, while refeeding mainly potentiates adipogenesis in subcutaneous depots [15]. In the present study, subcutaneous fat was the only depot in which there was sufficient quantity to sample for molecular analysis, thus it is unclear how various depots are affected by short-term changes in nutritional status in young quail.

Although there is limited knowledge on adipose tissue metabolism in quail chicks, our group reported that subcutaneous adipose tissue metabolism is highly dynamic in both broiler [1, 16] and body weight-selected genetic line [6] chickens during the early post-hatch stage. Changes observed in plasma NEFAs together with the unchanged expression of genes involved in lipolysis (melanocortin receptor 3 and monoglyceride lipase) with or without refeeding suggest that the subcutaneous depot in quail chicks, like that in adult mice, is not preferred for lipid mobilization during fasting [15]. It should be noted, however, that a small subset of genes were measured in the present study and not all would be expected to change at the transcriptional level within the time period that was assessed.

In adult mice, refeeding for 24 hours following a 72-hour fast increased expression of C/ebpα in the subcutaneous depot (inguinal) together with proliferator-activated receptor γ (Ppar γ) and sterol regulatory element-binding protein 1c (Srebp1c), which are involved in adipogenesis [15]. In the subcutaneous depot of 21-day broilers, neither 24 hours of fasting nor 4 to 24 hours of refeeding had an effect on the expression of fatty acid binding protein 4 (FABP4) [14], which facilitates lipid uptake and the maturing of adipocytes [17]. In the present study, there was
decreased expression of C/EBPα with unchanged FABP4 mRNA in the subcutaneous depot of fasted quail, regardless of refeeding treatment, indicating reduced adipogenesis in the absence of exogenous nutrition. This gene expression pattern is similar to that in 4-day old broilers where fasting for 3 hours with or without 1 hour of refeeding reduced the mRNA abundance of adipogenesis regulators C/EBPα, PPAR γ and SREBP1 without altering the expression of FABP4 [1].

Neuropeptide Y (NPY) and its receptor NPY2R, which are involved in promoting adipogenesis in chicken preadipocytes [18, 19] did not change at the mRNA level. However, we observed that expression of NPY5R was reduced in fasted quail after refeeding. Currently there is no report on NPY5R expression in the adipose tissue of Japanese quail. NPY5R mediates adipogenesis in 3T3-L1 cells [20] and our group [21] observed high expression of NPY5R mRNA in abdominal adipose tissue of chickens selected for high body weight. Thus, decreased NPY5R mRNA may relate to changes in adipogenesis induced by fasting and refeeding.

Conclusions

Six hours of fasting induced adipose tissue lipolysis and changes in gene expression of adipocyte-associated factors in young quail, and 1 hour of refeeding was sufficient to restore some of these changes to baseline. Such information is useful for understanding how early post-hatch nutrition influences adipose tissue development in avian species.
References


Table 6.1. Primers for real-time PCR.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer Sequence (5′-3′); Forward/Reverse</th>
<th>Accession NO.</th>
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<td>C/EBPα</td>
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<tr>
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<td>CAGAAGTGGGATGGCAAGAG / CCACCAGGTTCCCATCCA</td>
<td>XM_015855897.1</td>
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<tr>
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<tr>
<td>NPY5R</td>
<td>GGCTGGCTTTGTTGGAAAA / CTGTCCTCTGCTTCGTTTTGT</td>
<td>XM_015861003.1</td>
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1C/EBPα, CCAAT/enhancer binding protein α; FABP4, fatty acid binding protein 4; MC3R, melanocortin 3 receptor; MGLL, monoglyceride lipase; NPY, neuropeptide Y; NPY2R, neuropeptide Y receptor 2; NPY5R, neuropeptide Y receptor 5.
Table 6.2. Adipose tissue mRNA abundance in fasted and refed quail.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>C/EBPα</th>
<th>FABP4</th>
<th>MC3R</th>
<th>MGLL</th>
<th>NPY</th>
<th>NPY2R</th>
<th>NPY5R</th>
</tr>
</thead>
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<tr>
<td>Fed</td>
<td>1.05 ± 0.09a</td>
<td>1.25 ± 0.20</td>
<td>1.19 ± 0.22</td>
<td>1.09 ± 0.53</td>
<td>1.03 ± 0.21</td>
<td>1.11 ± 0.22</td>
<td>1.09 ± 0.32ab</td>
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<tr>
<td>Fasted</td>
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<td>1.56 ± 0.25</td>
<td>0.61 ± 0.28</td>
<td>1.52 ± 0.63</td>
<td>0.99 ± 0.25</td>
<td>1.60 ± 0.27</td>
<td>2.05 ± 0.37a</td>
</tr>
<tr>
<td>Refed</td>
<td>0.62 ± 0.11b</td>
<td>0.98 ± 0.25</td>
<td>0.86 ± 0.30</td>
<td>0.63 ± 0.63</td>
<td>1.07 ± 0.25</td>
<td>0.82 ± 0.27</td>
<td>0.45 ± 0.40b</td>
</tr>
<tr>
<td>P-value</td>
<td>0.0002</td>
<td>0.27</td>
<td>0.29</td>
<td>0.62</td>
<td>0.97</td>
<td>0.13</td>
<td>0.02</td>
</tr>
</tbody>
</table>

At 7 days post-hatch, Japanese quail were either continuously fed (Fed), fasted for 6 hours (Fasted), or fasted and refed for 1 hour (Refed), after which subcutaneous adipose tissue was collected for measuring mRNA abundance of the following genes via real time PCR: *C/EBPα*, CCAAT/enhancer binding protein α; *FABP4*, fatty acid binding protein 4; *MC3R*, melanocortin 3 receptor; *MGLL*, monoglyceride lipase; *NPY*, neuropeptide Y; *NPY2R*, neuropeptide Y receptor 2; *NPY5R*, neuropeptide Y receptor 5. All data are presented as means ± SEM (n = 8-12). a,bP < 0.05 (Tukey’s test).
Figure 6.1. Body weight of quail continuously fed (Fed), fasted for 6 hours (Fasted) or fasted for 6 hours followed by 1 hour of refeeding (Refed). All data are presented as means ± SEM (n = 8).
Figure 6.2. Plasma non-esterified fatty acid (NEFA) concentrations. Quail were continuously fed (Fed), fasted for 6 hours (Fasted) or fasted for 6 hours followed by 1 hour of refeeding (Refed). All data are presented as means ± SEM (n = 10). a,bP < 0.05 (Tukey’s test).
Chapter 7: Epilogue

The line chickens have been selected for low and high body weight from the same foundation population for 63 generations. The LWS are lean and hypophagic, with limited fat deposition and delayed ovulation, or even no ovarian development, while HWS are fat and hyperphagic, with excessive accumulation of fat and development of metabolic syndromes in the absence of feed restriction. These characteristics are similar to the clinical diagnoses of anorexia and obesity in humans, which makes the line chickens a valuable model to study physiological changes in anorexia and obesity. Moreover, the fact that chickens and humans share similar anatomy of abdominal and subcutaneous adipose tissues, and that both chickens and humans use liver, but not adipose tissue, as the main site for *de novo* lipogenesis makes chicken a more suitable animal model to study adipose tissue physiology compared to rodent models. In this dissertation, we compared the adipose tissue physiology of line chicks during the first two weeks post-hatch. While LWS had intense mobilization of body fat reserves from day of hatch to day 4 post-hatch and then a less than 2 times increase in body weight and less than 12% of total body fat mass, HWS had rapid growth throughout the first 14 days post-hatch and achieved over 5 times increase in body weight and more than 20% of body fat. Moreover, lipid mobilization was more intensive from subcutaneous than other depots during the first 4 days post-hatch regardless of line whereas fat accumulation was more significant in clavicular and abdominal depots in HWS from day 4 to day 14 post-hatch. It is worth noting that there was a significant three-way interaction on *NPY* expression and all two-way interactions were significant for *NPYR2* expression. Previous research in our group not only confirmed the potent orexigenic effect of NPY in chickens, but also showed its adipogenic effects on chicken preadipocytes, which is likely mediated through NPYR2 according to mammalian studies. To test this hypothesis, we
further attempted to determine the effect of NPY on NPYR2 knockdown chicken preadipocytes by using a lentivirus-mediated transduction method. However, there was a dilemma—the transduction was only successful at high multiplicity of infection (MOI) whereas at high MOI, there was not sufficient cell numbers for downstream experiments and there was a loss of GFP expression with prolonged cell culture (Figure B.1). Because there is no immortalized adipogenic cell line like murine-derived 3T3-L1 available from chickens, and only lentiviral-based transduction can integrate the target sequence into the host genome to achieve long-term knockdown compared to other viral vectors or siRNA-based methods, we failed to generate NPYR2 knockdown cells for downstream proliferation and differentiation experiments. Further efforts may be made to develop chicken-derived immortalized adipogenic cell lines or to adopt CRISPR-based methods for effective gene editing in chicken primary cells. As a continuation of the tissue-level studies, we further compared the cellular level differences between lines in SVF and adipocyte compartments. We only determined the gene expression pattern in the subcutaneous depot at hatch and in the abdominal depot at day 14 post-hatch because of the considerable differences observed from the tissue-level study. LWS had higher expression of adipocyte precursor cell markers and genes involved in cell proliferation, fatty acid oxidation and apoptosis, but lower expression of genes involved in lipid uptake compared to HWS. These results suggested intensive apoptosis of the adipocyte precursor cells as a novel explanation of reduced adipogenic potential and lack of fat accumulation in chicks predisposed to anorexia during early life.

The extension of the cellular and molecular adipose tissue physiology study from older line chickens to development during early post-hatch growth may provide valuable insights in the pathogenesis of AN and obesity at early ages in humans, especially considering that so far, no
such study has been done in childhood AN, which may be attributed to the difficulty in
diagnosis, limited availability of biopsies, as well as ethical issues. However, the following
considerations should be noted when referring to the results from chicken models:

1) As mentioned in the literature review, the prevalence of AN in adolescent and adult males is
much lower than in age-matched females, whereas before puberty, AN incidence in boys, though
still lower than that in girls, is much greater compared to post-pubertal males [1]. Our study
showed no sex difference in adipose tissue metabolism regardless of line during the first 14 days
post-hatch, while older birds have significant difference within and between lines [2]. Although
both the chicks used in our experiment and children with early onset AN are at ages prior to
sexual maturity, it is not known which childhood developmental stage is comparable to the early
post-hatch chicks. There is the possibility that adipose tissue metabolism in pre-pubertal children
presents sex differences as early as 7 years old, which was the earliest reported age of childhood
AN [3].

2) Leptin is a critical adipokine produced by adipose tissue in humans. It is secreted in
proportion to body fat mass and plays an essential role in regulating energy homeostasis.
However, not until recently was this gene found in chickens (bearing a low homology to the
human sequence) and compared to its intense expression in human adipose tissue, the expression
in chicken adipose tissue is negligible, and highest expression was in the cerebellum,
hypothalamus and cerebrum [4]. Interestingly, expression of RBM28, a neighboring gene of
leptin found in diverse vertebrate genomes, was intense in chicken adipose tissue and higher in
lean than obese chickens, whereas no such difference was found in humans, nor difference in
leptin expression in adipose tissue of lean and fat chickens. Therefore, alternative signaling
pathways for energy homeostasis other than leptin may be recruited in chickens due to evolutionary adaptations.

3) Chickens are precocial animals with yolk as the main energy source during the initial days post-hatch and are able to feed themselves at hatch. Therefore, adiposity during the early post-hatch stage of chicks may be affected by yolk utilization efficiency and the availability and conversion ratio of feed. In contrast, in humans, the early onset of eating disorders or adiposity is often related to maternal and family influences [5].

4) Consistent with the previous comprehensive research in commercial broilers and layers [6], abundant and metabolically dynamic clavicular fat in line chicks was found during the early post-hatch stage. However, to the best of our knowledge, the function of clavicular fat in chickens remains unclear. Although there is supraclavicular fat in humans, the fact that humans and birds share different anatomy in the clavicle (fused so-called “wishbone”/furcula in birds and non-fused so-called “collarbone” in humans) and the fact that the supraclavicular depot is a reservoir of activated BAT in both human infants and adults [7, 8] whereas chickens have no reported BAT and depend on avUCP (lack the ortholog to UCP-1)-mediated non-shivering thermogenesis in muscles [9] make it unlikely that there is a functional link between the depots in these two species.

5) Apoptosis was increased in subcutaneous adipose tissue of obese humans [10] and in the epididymal depot of diet-induced obese mice [11]. In contrast, steers with low body weight gain had greater GZMA mRNA abundance in mesenteric adipose tissue than those with higher body weight gain, while the underlying mechanism was not explained [12]. To date, there is no report on GZMA-mediated adipocyte (precursor cells) apoptosis, nor its relation to leanness in any
species. Moreover, the apoptotic function of GZMA has only been studied in chicken immune cells [13]. Whether the highly expressed GZMA in LWS chicks can explain their leanness requires further research to confirm.

In continuation of our previous research on stress-induced alteration of appetite regulation in LWS chicks, studies in Chapter 5 further determined the involvement of epigenetic modifications in stress-induced gene expression changes in LWS. Results from global DNA methylation and DNMT activity indeed confirmed epigenetic changes in the ARC, but not PVN in response to early post-hatch stress exposure. Meanwhile, gene-specific analysis further revealed less DNA methylation in the promoter region of CRF in the PVN, which contains the core binding sites for MBD2 to repress gene expression. These results suggest a novel target towards stress-induced appetite dysregulation in anorexia, while certain limitations should be considered:

1) As mentioned above, our aim was to determine the involvement of epigenetic modifications in early-life stress-induced gene expression changes. Therefore, we did not distinguish 5-mC versus 5-hmC, nor determined the activity of specific DNMTs. And the results indicate that the methylation changes of specific genes are not necessarily reflected by global DNA methylation and DNMT activity changes.

2) This study only focused on NPY in the ARC and CRF in the PVN of LWS chicks because of their significant gene expression change reported in the previous study. Other genes, whether involved in appetite regulation / stress response or not, may also be influenced by stress exposure. This is also reflected by the non-matched global methylation result versus the gene-specific methylation results. In addition, this study only determined the methylation status of
CpG-enriched regions in the promoter of *NPY* and *CRF* genes. In the liver and muscle of broilers and red jungle fowl, promoter regions are lowly methylated and negatively correlated with gene expression [14], which is consistent with our findings here. Meanwhile, highly methylated regions were found in the intergenic regions (between annotated 3’ and 5’ ends of genes) followed by introns and exons [14]. To explore deeper into the metabolic pathways affected by the stressors, integrated data from whole-genome DNA methylation profiling and RNA sequencing is needed.

3) Only DNA methylation was determined in this study as it is the most well studied epigenetic modification among species. To further confirm the mechanism involved in the change in *NPY* expression upon stress exposure, epigenetic modifications by histone and non-coding RNAs may be studied in future research.

Upon stress exposure with a combination of cold and delayed access to feed, one would expect increased energy consumption and thus an increased energy requirement for compensation. Yolk as the major source for energy in early post-hatch chicks is expected to be intensively and rapidly utilized. However, stressed LWS chicks had even more yolk remaining than those non-stressed counterparts. Instead, we found that both fat and lean masses were lower in stressed chicks, which together contributed to a lower body weight, indicating the sacrifice of growth performance for the compensation of energy requirements upon stress exposure early post-hatch. Research in humans and animals frequently link early-life stress to alterations in metabolism in adulthood. This is the first study reporting body composition changes at an early stage of life after stress exposure. It would be a fruitful avenue to further determine the body composition changes as well as the developmental and metabolic alterations of adipose tissue, skeletal muscle and gastrointestinal tract at later life stages of the stressed LWS to integrate the knowledge of the
long-term effect of early post-hatch stress on energy homeostasis. Moreover, it is logical to determine if feeding diets rich in methyl donors, for instance, wheat germ and soybeans, could mitigate metabolic changes induced by early post-hatch stress exposure and thereby improve outcomes later in life. Such research may also provide insights into the critical time point at which to modulate metabolic changes through dietary or lifestyle interventions.

In Chapter 6, we report the effect of short-term fasting and refeeding on adipose tissue metabolism in young Japanese quail. Plasma NEFAs were increased by 6-hours of fasting, similar to fasted chicks and adult quail, and values rapidly recovered to normal levels within 1 hour of refeeding. Interestingly, alterations in gene expression within the subcutaneous depot was similar to fasted chicks but different from fasted adult mice. Meanwhile, reduced NPY5R expression after refeeding was observed in the quail. Quail as a less artificially selected avian species compared to chickens, while more lab-adapted than a bird from the wild, may be a good model to study metabolic similarities and differences among avian species, as well as between avian and mammalian animals for evolutionary perspective. Indeed, research has already found high synteny conservation between quail and chicken [15], as well as similarities in genes involved in behaviors and diseases between quail and human [16]. Nevertheless, it should be noted that age, sex and sensitivities to physiological changes, etc. may contribute to the discrepancies in physiology between quail and other animal species. Thus, comparisons should be made with caution.

In summary, research in this dissertation expanded current knowledge of adipose tissue physiology in avian species during the early post-hatch stage from both cellular and molecular perspectives and we proposed novel molecular mechanisms of early-life stress-induced epigenetic modifications related to appetite regulation in anorexia. These findings may benefit
the in-depth understanding of the pathogenesis of anorexia and obesity and help others identify therapeutic targets as pharmacological strategies to recover energy homeostasis.
References

Appendix A

Figure A.1. *In situ* hybridization to determine the distribution of *NPY* mRNA in abdominal adipose tissue of 14-day old LWS chicks. The procedure was conducted with RNAscope 2.5 HD Detection Reagent-RED (ACD, CA, USA) with the negative control (A-C) targeting a bacterial gene *dapB* to assess non-specific binding, and positive control (G-I) targeting the housekeeping gene *PPIB*. Custom-designed *NPY* probe was used to localize the mRNA expression of *NPY* (D-F). Images were captured with a Nikon DS-Ri1 high-resolution microscope camera using 400× magnification under bright field (A, D, G), TRITC channel (B, E, H) and overlay (C, F, I).
Figure A.2. *In situ* hybridization to determine the distribution of *NPY* mRNA in abdominal adipose tissue of 14-day old HWS chicks. The procedure was the same as described in Figure A.1. A-C: Negative control; D-F: NPY; G-I: Positive control.
Appendix B

Figure B.1. NPYR2 knockdown mediated by lentiviral-shRNA transduction in chicken preadipocytes. Images were captured with an EVOS® FL Imaging System under 100× magnification for green fluorescent protein (GFP). GFP signals were detected in both scrambled-shRNA control lentiviral transduced cells (A, merged in bright field on the right column) and NPYR2-shRNA lentiviral transduced cells (B, merged in bright field on the right column). Preadipocytes isolated
from chicken abdominal adipose tissue were cultured in DMEM/F12 with 10% defined fetal bovine serum and 1% penicillin/streptomycin until 80% confluence in 24-well plate. Cells in each well were then randomly assigned to be transduced with scrambled control lentiviral particles (pLenti-shControl-GFP; Welgen Inc., MA, USA) or NPYR2-containing lentiviral particles (pLenti-shNPYR2-shRNA-GFP) with 8 μg/mL polybrene (Sigma, MO, USA) at multiplicity of infection (MOI) = 10,000. According to the preliminary trials, this is the lowest MOI with successful and sufficient GFP expression in scrambled control cells without alteration in cell proliferation. Such high MOI has also been used to infect human and murine primary cells [1]. However, compared to the scrambled control (A), the NPYR2 knockdown cells (B) showed lowered transduction efficiency and slow proliferation. Moreover, cells transduced with shNPYR2 had morphology changes (C) compared to those controls, which has also been reported in lentiviral based gene knockdown in other cell types [2]. To acquire enough successfully transduced cells for downstream experiments, cells were maintained until 20 days post-transduction, nevertheless, along with the decreased number of cells expressing GFP (B), no difference in NPYR2 gene expression was observed between the control and NPYR2 knockdown cells (D). The successful expression of GFP in both scrambled control and NPYR2 knockdown cells indicate no difficulty of lentiviral particles entering chicken preadipocytes. The difference in GFP expression/transduction efficiency and cell proliferation between the control and knockdown groups may result from the disruption of the chicken genome upon knockdown, as the scrambled control contains a random sequence not binding to chicken genome, whereas shNPYR2 binds to the NPYR2 sequence in chicken genome to suppress gene expression.
References
