PATHWAY PROFILING IDENTIFIES MECHANISMS OF ADIPOSE DEPOSITION IN DOMESTIC CHICKENS

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Bo Ji

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DEDICATION

This dissertation is dedicated to my family.

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ABSTRACT

The domestic chicken is an attractive, but underutilized, animal model for studies of adipose tissue biology, metabolism and obesity: 1.) like humans, chickens rely on liver rather than adipose tissue for the majority of *de novo* lipogenesis; 2.) quantitative trait loci (QTLs) linked to fatness in chickens contain genes implicated in human susceptibility to obesity and diabetes; 3.) chickens are naturally hyperglycemic and insulin resistant; and 4.) a broad selection of genetic models exhibiting a range of fatness are available. To date, however, little is known about regulation of adipose metabolism in this model organism.

Affymetrix arrays were used to profile gene expression in abdominal adipose tissue from broiler chickens fed *ad libitum* or fasted for five hours. Quantitative real time polymerase chain reaction (QPCR) was used to validate microarray results for select genes. A total of 1780 genes were differentially expressed in fasted vs. ad libitum fed ($p<0.05$) tissue after correction for multiple testing. Gene Ontology and pathway analyses, combined with Western blot validation, indicated significant effects on a broad selection of pathways related to metabolism, stress signaling and adipogenesis. In particular, fasting upregulated rate-limiting genes in both the mitochondrial and peroxisomal pathways of beta-oxidation. Enhanced fatty acid oxidation in white adipose tissue was further suggested by a significant increase in tissue content of the ketone beta-hydroxybutyrate. Expression profiles suggested that, despite the relatively brief duration of feed withdrawal, fasting suppressed adipogenesis; expression of key genes in multiple steps of adipogenesis, including lineage commitment from mesenchymal stem cells, were

significantly down-regulated in fasted vs. fed adipose tissue. Interestingly, fasting increased expression of several inflammatory adipokines and components of the toll-like receptor 4 signaling pathway. A second study with Affymetrix microarrays of Fayoumi, Leghorn and broiler adipose tissue revealed that genetic leanness shared molecular signatures with the effects of fasting. In supervised clustering analysis, fasted broiler chickens clustered with lean Fayoumi and Leghorn lines rather than with the fed broiler group, suggesting that fasting manipulated expression profiles to resemble those of the lean phenotype.

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SELECTED ABBREVIATIONS

AA	Arachidonic acid
AMPK	AMP-activated protein kinase
ANOVA	Analysis of variance
BCAAs	Branched chain amino acids
Camp	Cyclic adenosine monophosphate
CEBPα	CCAAT, Enhancer-binding protein alpha
CPT1	Carnitine palmitoyltransferase 1
DGAT2	Diacylglycerol O-acyltransferase homolog 2
DHA	Docosahexaenoic acid
EPA	Eicosapentaenoic acid
FDR	False discovery rate
GO	Gene ontology
IRS-1	Insulin receptor substrate-1
IRβ	Insulin receptor β-subunit
LXR	Liver X receptor
MSC	Mesenchymal stem cell
NADPH	Nicotinamide adenine dinucleotide phosphate
NEFA	Non-esterified fatty acid
PD	Pyruvate dehydrogenase
PDGF	Platelet derived growth factor
PDK4	Dehydrogenase kinase 4
PGB	Preproglucagon class B transcript
PI3K	Phosphatidylinositol 3-kinase
PPAR,	Peroxisome proliferator-activated receptor
PUFA	Polyunsaturated fatty acids
QPCR	Quantitative PCR
Shc	Src homology 2 domain-containing substrate
SREBP	Sterol regulatory element-binding protein
TAG	Triacylglycerol
TLR	Toll-like receptor
TNF	Tumor necrosis factor
VLDL	Very low density lipoprotein
WAT	White adipose tissue

CHAPTER I

LITERATURE REVIEW

Introduction and Objectives

The broiler industry has experienced decades of rapid expansion due to population growth and the public's preference for white chicken meat, creating a ~$26 billion broiler market in 2012 in the United States [1]. Broiler production now exceeds that of both beef and pork, and broilers are the most efficient of the major food animals. Intensive genetic selection for rapid growth has allowed the broiler industry to keep up with the demand. However, it has also resulted in undesirable traits, including an increasing fat content. Carcass fat in broilers at market age accounts for 10-15% of the total carcass weights [2]. Excess fat is an economic loss to the broiler industry because it wastes feed by its conversion to adipose rather than lean tissue and because of costs incurred from the need to dispose of fat after processing. Excess fat may also contribute to the development of detrimental traits arising from genetic selection for rapid growth, such as reduced fertility and immunocompetence, both of which are seen in obese humans [3]. The broiler industry requires new strategies to reduce the fatness in broilers, which would economically benefit producers and likely improve health and welfare of chickens. Such strategies require the knowledge of adipose tissue metabolism and biology, which is still limited in chicken.

The overall objective of this dissertation was to identify the mechanisms that regulate fat deposition in domestic chickens using an approach that couples comprehensive transcriptomic and metabolomic methods with system and cellular physiology. The objective of study 1 was to characterize the effects of energy restriction

and insulin on adipose tissue in broiler chickens. The objective of study 2 was to identify functional gene and metabolite classes differentially expressed in adipose tissue of lean (Fayoumi and Leghorn) and broiler chickens. The objective of study 3 was to identify the similarity between lean chickens and the fasted broilers. The data from study 1 & 2 were then integrated together using statistical model to identify the similarity between fasted broilers and lean chickens.

The findings of this work will provide comprehensive information to identify potential new targets for genetic selection or management strategies to reduce fat accumulation in commercial broilers and to further develop chicken as a model organism for studies of human obesity.

Domestic Chickens

Domestic chickens originated from their wild ancestor, red jungle fowl, about 8,000 years ago in Southeast Asia according to the archaeological evidence [4]. Chickens exhibited genetic adaptation to a new environment morphologically, physiologically and behaviorally during long term domestication [5]. In recent decades, chickens have been subjected to human-driven selection mainly for food purposes by laying eggs or providing meat, leading to remarkable phenotypic changes.

Selection driven by the poultry meat and egg industries resulted in a decrease in the total number of chicken breeds and produced the breeds that currently dominate the world's poultry industry, especially between 1930 to 1950 [6]. Layers were selected by simultaneous improvement of multiple traits, including the age at sexual maturity, livability, egg number, shell strength, egg size, and egg weight [7]. Therefore, the dominant egg-laying lines used commercially today, including White Leghorns, Golden

Comets and Rhode Island Red, are characterized by quick maturity, low mortality, small body size, high production of saleable eggs, low feed cost per egg production and optimal egg quality. For example, feed conversion of layers has improved from 2.95 to 2.01 g feed per gram egg, from 1960 to 2001[8], and egg production has increased from 176 to 309 eggs per hen per year from 1925 to 1998.

Today's meat type chickens mostly refer to broilers. Modern broilers with superior meat production performance are based heavily on a crossbred of White Cornish, New Hampshire and White Plymouth Rock. They exhibit features of fast growth, efficient feed conversion and high yield of breast muscle [2]. Their parental lines, White Cornish, New Hampshire and White Plymouth Rock, are dual-purpose breeds for both egg and meat, and have inferior meat production but better egg production compared to broilers.

The broiler and egg industry has established an efficient and sustainable industry by using genetic improvement. The U.S. Department of Agriculture (USDA) has forecast that 37.3 billion pounds of broiler meat with the value of $27.7 billion will be produced in 2013 according to the USDA Livestock, Dairy and Poultry Outlook. Likewise. total egg production will be about 7.5 billion pounds.

Genetic Selection

Modern commercial broiler breeding was initially developed in the United States as a result of selection for rapid growth. In 1945, the American grocer A&P (Atlantic & Pacific Tea Company) organized the national "Chicken of Tomorrow" contests to encourage meat-type poultry breeders to develop a more efficient broiler [9]. The genetic selection criteria were initially based on the weight and growth rate of birds, which were stable and heritable traits and helped improve broiler breeder performance. As feed costs

rose, the industry switched the selection criteria to feed efficiency, which helped to produce chicks cost effectively. Genetic selection has greatly improved chicken production over the last 50 years and provided high quality dietary protein to humans. The market age of today's broiler is about 6 weeks compared to 12 weeks in 1950s [10]. It only needs 3.4 kg of feed in less than 40 days to produce a 2.0 kg broiler today, in contrast to 7.2 kg of feed in 95 days to produce a 1.3 kg broiler before genetic selection [3].

However, intensive genetic selection for rapid growth inadvertently selected for increased carcass fat. Carcass fat in broilers at market age accounts for 10-15% of the total carcass weight and deposits at a rate of up to six grams fat/kg body wt/day between 42 and 49 days of age [2, 11, 12]. This is probably because abdominal fat is proportionately largest when body weight and growth rate are the critical traits selected, since it has a higher heritability than skeleton. Also, the broiler tends to overconsume feed, which is another reason for fat deposition in broiler chickens. Excess fat is an economic loss to boiler industry by reducing the conversion of feed to meat, and the disposal of fat leads to additional economic loss. Excess fat may also contribute to the development of other detrimental traits, such as reduced reproductivity and immunocompetence, which consequently affect growth and yield of meat [3].

To solve these problems, broiler breeder management programs developed feed restriction methods, i.e., skip-a-day feeding regimen, to manage broiler breeders [13]. In this practice, from 21 to 140 days, broiler breeders are fed two's days' worth of feed on one day and no feed on the next day. In total, the same amount of feed is provided but the feed is rapidly consumed, producing a window of fasting. This has proven to be an

effective approach to control excess fat deposition in broiler chickens by altering hepatic lipogenic gene expression and abdominal fat content [14-17]. However, feed restriction is not the ultimate solution, because it is associated with reduced body weight and meat production, hunger stress and impaired welfare [18]. Skip-a-day program is practiced widely in the USA, but there are some restrictions and regulations already in place to limit the application of this program. In particular, its use is banned in some European countries, e.g., in the UK. The US is expected to face the challenge to address these welfare issues in the coming years.

Therefore, a successful strategy that can reduce fat deposition without compromising lean meat growth is required. Unlike humans, increasing energy expenditure by increasing exercise is not an option for chickens. A successful strategy, either by genetic selection or by diet and nutrient management, would alter energy portioning, preferentially deposit into lean tissue or increase utilization of fat for energy. The fact that there are both naturally leaner and fattier lines of chickens among the spectrum of common breeds illustrate that there are mechanisms that affect fatness in chickens. However, little is known about these mechanisms. Thus, it would be of particular interest to understand the cellular and molecular pathways leading to fat loss by feed restriction or nutrient deprivation, and to further manipulate the genes associated with adiposity in broiler chickens.

Several genetic lines of fat and lean chickens have been developed through different phenotypic selection strategies. These lines have been used to study the relationship between lipid metabolism, fatness and rapid growth. The lines selected by Siegel for 8-week high and low body weight are representative models to study the

correlated traits associated with rapid growth. High body weight chickens showed increased feed efficiency, feed consumption, increased carcass fat, increased muscle size and number, egg size, but decreased plasma growth hormone, immunocompetence and egg production [3]. Lean broiler chickens with reduced abdominal fat and body lipids were also obtained through selection on higher feed efficiency (feed consumed/weight gain), which confirmed high negative correlation between feed efficiency and fatness [19].

Genetic lines have also been selected for plasma levels of very-low-density lipoprotein (VLDL). The high VLDL line of chickens had 38% difference in total lipids and 49% more abdominal fat weight, but there were no differences in body weight, food intake, hepatic lipogenesis, and lipoprotein lipase (LPL) activity in the high VLDL line compared to the low VLDL line [20]. Similarly, selection for low plasma glucose level resulted in fat chickens [21]. These studies clearly demonstrated the relationship between lipid and carbohydrate metabolism and fatness.

Direct selection specifically on low abdominal fat content effectively resulted in leaner broiler chickens [22]. The fat line chickens that resulted from this selection criteria had four times more abdominal fat and 72% more lipids than lean line chickens, but no difference in body weight or food intake [22]. Hepatic lipogenesis, VLDL secretion, SCD (Δ9-Desaturase) activity, and adipocyte size and number were also higher in fat line chickens [23, 24]. Collectively, fat line chickens produced through various genetic selection criteria showed common features, including high plasma levels of VLDL, low plasma levels of glucose, high hepatic lipogenesis and adipocyte size and number. These

results reflected importance of hepatic lipogenesis and glucose consumption in fat accumulation.

Adipose Tissue Biology

White adipose tissue & brown adipose tissue

In mammals, there are two major types of adipose tissue: white adipose tissue and brown adipose tissue. White adipose tissue, the most common type of adipose tissue, is characterized by large lipid droplets in which triglyceride are stored. Brown adipose tissue is “brown” because of a high density of mitochondria. The hallmark of brown adipose tissue is expression of mitochondrial uncoupling protein-1 (UCP-1), which uncouples proton transfer in mitochondria from Adenosine-5'-triphosphate (ATP) generation, and the dissipation of energy as heat rather than its storage in ATP. White adipose tissue can be further classified as subcutaneous and visceral fat, according to the location in the body [25]. Subcutaneous fat is located beneath the skin and visceral fat is adipose tissue that lies around the central organs. The predominant site of adipose deposition in chickens is abdominal fat and, to a less extent, subcutaneous fat and intramuscular fat. Chickens have relatively low intramuscular fat compared to other agricultural animals [26], and no brown adipose tissue, which differs from rodents (present throughout life) and humans (present at birth, reduced in adults) [27].

Adipogenesis

White adipose tissue is composed of mature adipocytes and cells of the stromal vascular fraction, including preadipocytes, mesenchymal stem cells (MSC), endothelial cells and immune cells, such as T cells and macrophages [28]. Adipocytes arise from mesenchymal stem cells (MSC), a multipotent stem cell that has the potential to differentiate into adipocytes and other tissues of mesenchymal origin. This is a two-step

process: preadipocytes are generated from MSC by lineage commitment and differentiate into mature adipocytes through early differentiation and terminal differentiation [29].

This process is orchestrated by a transcriptional cascade involving the peroxisome proliferator-activated receptor gamma (PPARγ),CCAAT-enhancer-binding protein alpha (C/EBPα) and sterol regulatory element binding protein-1c (SREBP1) [30-32]. PPARγ is a member of the nuclear receptor superfamily and is activated by a variety of fatty acids and their derivatives, such as prostaglandins. PPARγ participates in adipogenesis through binding as obligate heterodimers with retinoic X receptor (RXR). C/EBP α is expressed late in adipogenesis and is a key regulator of adipocyte differentiation by regulating expression of adipogenic genes. SREBP1 is the transcription factor that is expressed in the early stage of adipogenesis and activates PPARγ by secreting lipid molecules that bind directly to PPARγ [33, 34].

The molecular steps of adipogenesis are comparable between chickens and humans. Retroviral transfection of C/EBPα, PPARγ and SREBP-1 into chicken embryo fibroblasts could induce embryo fibroblasts differentiation into adipocyte-like cells associated with lipid droplets accumulation and increased A-FABP [32]. A recent study pointed out that fatty acid is essential to induce adipocytes differentiation and identified regulatory factors that affect glycerol-3-phosphate dehydrogenase (GPDH) activity, intracellular triglyceride accumulation and adipogenesis in chicken, including expression of PPARγ and fatty acid-binding protein (aP2) [35].

Adipose Tissue Metabolism

Adipose tissue is the major site for energy storage in the form of triglyceride. A triglyceride molecule consists of three fatty acids esterified to a glycerol backbone. Glycerol is produced from glucose through glycolysis and glyceroneogenesis. In mammals, fatty acids can come from diet or be synthesized from glucose in lipogenic tissue by catalyzed by fatty acid synthase. Dietary fatty acids are absorbed by enterocytes, packaged with cholesterol, lipoproteins and other lipids into chylomicrons, and transported into circulation. Blood-borne chylomicrons are rapidly disassembled, utilized or stored in adipose tissue. Lipids synthesized in the liver are packaged into VLDLs and released into the blood directly. TAG-rich VLDL reaching the surface of adipose tissue vascular are targets of lipoprotein lipase (LPL) and release fatty acids, which are taken up by adipocytes.

In chickens, the contribution of portomicrons (similar to chylomicrons in mammals) from dietary fats to triglyceride is low, since there is about 5% of dietary lipids in regular feed [36]. Triglyceride (TAG) deposits in chicken adipose tissue mainly through the uptake of fatty acids from circulating TAG-rich VLDL delivered by the liver. Increased VLDL levels were found in virtually all lines of chickens associated with high fatness, reflecting the critical role of the liver in fatness of chickens [24]. The contribution of *de novo* lipogenesis in liver to fat deposition was also confirmed using the chicken lines selected by plasma VLDL concentration [20]. Low VLDL line chickens were found to be associated with a relative decrease in

abdominal fat and total body lipid and lipogenic enzyme activity (FASN, ME, ACL) and an increase in body protein [37].

In the refed state after fasting, both lean and fat line chickens showed elevated plasma VLDL concentration (14-fold and 7-fold, respectively), reaching similar levels at last, which indicated that hepatic conversion of glucose to fatty acids is common in all lines of chickens, and nutrient status may induce a compensating increase of plasma VLDL in lean line chickens Fat line chickens also showed higher hepatic VLDL removal rate, other than VLDL secretion [38]. Accordingly, increased adipocyte number and size result in higher fatty acid uptake rate in adipose tissue [23]. Fat line chickens showed higher LPL activity and its activity was found to be positively correlated with fat deposition in broilers [23]. Greater LPL activity per cell was observed not only in genetically fat line chickens, but also in broiler chickens compared to layers, which exhibited a lower growth rate in both abdominal adipose mass and body mass [39].

***De novo* lipogenesis**

De novo lipogenesis is an enzymatic pathway that converts acetyl-CoA, the intermediate of dietary carbohydrate metabolism, to fatty acids. There are three types of fatty acids based on the number of double bonds, i.e., saturated (SFA, no double bond), monounsaturated (MUFA, one double bond) and polyunsaturated fatty acids (PUFA, two or more double bonds). *De novo* lipogenesis begins with the condensation reaction of acetyl-CoA with malony-CoA to produce six-carbon intermediates and proceeds by the serial addition of two-carbon units to synthesize 16-carbon palmitate. Palimitate undergoes elongation and desaturation (the addition of double bonds) process, leading to the formation of various PUFAs. PUFAs can be classified into two categories, the n-3

and n-6 series, depending on the position of the final double bond nearest the terminal methyl group. Eicosapentaenoic acid [20:5 (n-3), EPA] and docosahexaenoic acid [22:6 (n-3), DHA] are the most common n-3 long-chain PUFAs. They exert beneficial functions, i.e., anti-inflammation, anti-obesity and reducing plasma triglycerides in humans and rodents. The general process of *de novo* lipogenesis is shown in Figure 1-1.

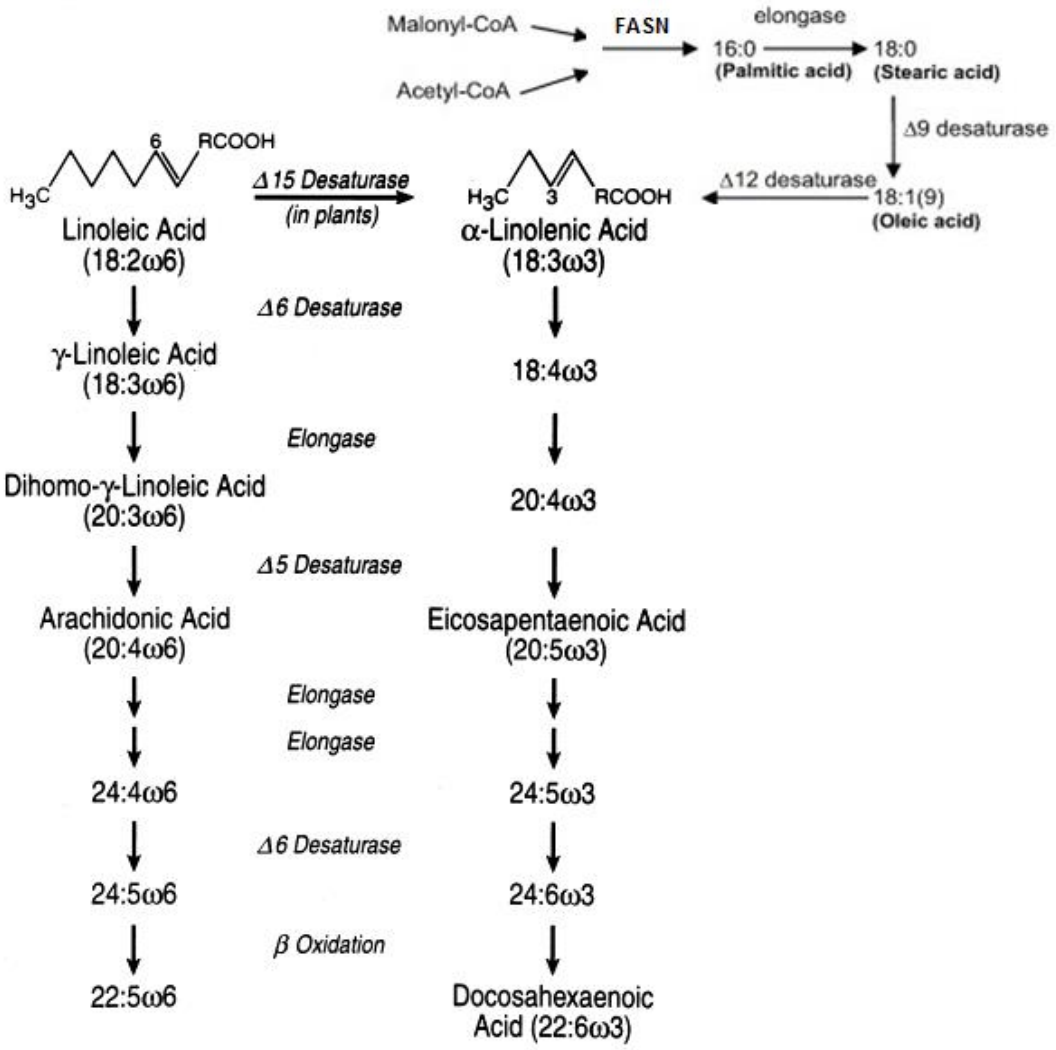


Figure 1-1. Pathway of fatty acids biosynthesis, elongation and desaturation[40]

De novo lipogenesis occurs in liver and adipose tissue in a species dependent manner. In rodents, adipose tissue accounts for about 50% *de novo* lipogenesis. Whereas in human and chickens, only 15% of *de novo* lipogenesis occur in adipose tissue, and the rest is from liver [41]. The product of *de novo* lipogenesis in liver are secreted in the form of VLDL and delivered to other tissues.

Acetyl-CoA carboxylase (ACC) is the rate-limiting enzyme catalyzing the irreversible carboxylation of acetyl-CoA to produce malonyl-CoA. Two major isoforms of ACC, ACACA and ACACB, are predominately expressed in adipose tissue and skeletal muscle, respectively. The other critical enzyme is fatty acid synthase (FASN), which catalyzes synthesis of palmitate from acetyl-CoA and malonyl-CoA. Fatty acids are subsequently esterified with glycerol into triglycerides [42]. Lipogenic enzymes, such as FASN, ME, ACC, ATP citrate lyase (ACL) and steroyl CoA (delta 9) desaturase 1 (SCD1), have relatively low activity in chicken adipose tissue [36, 43]. Expression of lipogenic enzyme genes is influenced by Sterol regulatory element binding protein-1 (SREBP1) [44, 45]. SREBP1 is more expressed in the liver than adipose tissue, which is also observed in humans. This may explain the limited *de novo* lipogenesis in adipose tissue of both humans and birds.

Although *de novo* lipogenesis in chicken adipose tissue is limited, it is regulated by nutritional manipulation. For instance, lipogenesis from acetate was inhibited by VLDL as an exogeneous source of fatty acids, which was also observed in mammalian adipose tissue [41]. Likewise, *de novo* lipogenesis may be enhanced when excess carbohydrates are consumed.

Fatty acid uptake, re-esterification and lipolysis

Long chain fatty acids translocate across the plasma membrane through passive diffusion, and this process is facilitated by membrane associated fatty acid transport protein (FATPs, SLC27 family). FATP expression in chicken adipose tissue showed an age- and depot-dependent pattern of expression. Expression in the abdominal depot increased with age to its highest level at eight weeks of age then decreased, whereas in subcutaneous fat, it reached a peak at 12 weeks of age[46]. This observation is consistent with the fact that birds are fatter at an older age.

Fatty acid esterification in adipocytes involves two steps. First, a fatty acid is activated to its CoA derivatives; second, fatty acyl-CoA is added to the glycerol "backbone" in adipocytes. The two steps are catalyzed by acyl-CoA synthetase (ACS) and diacylglycerol acyltransferase 2 (DGAT2), respectively. During this process, glycerol-3-phosphate (G3P) is formed by phosphorylation of glycerol or synthesized from pyruvate or lactate, which is catalyzed by phosphoenolpyruvate carboxykinase (PEPCK). The incorporation of G3P into TAG is catalyzed by glycerol kinase (GK), which is considered absent in adipose tissue. However, the mechanism and regulation of these processes in chicken adipose tissue remains unknown.

In mammals, the key enzymes involved in lipolysis of TAG in adipose tissue include hormone sensitive lipase (HSL) and adipose triglyeride lipase (ATGL). However, the existence of HSL has not been identified in chickens [47]. In mammalian adipocytes, HSL was found to form a complex with fatty acid binding proteins (FABPs), which indicated the lipolytic effect of FABPs. A-FABP overexpression in chicken adipocytes was found to be associated with lipid accumulation [48]. Adipose triglyceride lipase (ATGL) is the rate-limiting enzyme in

TAG lipolysis, and its expression in chicken abdominal adipose tissue is high. A recent study showed that dexamethasone increased ATGL expression through activation of glucocorticoid [49], and 24 hours of fasting increased ATGL expression at both mRNA and protein levels in broiler adipose tissue [50]. Moreover, ATGL gene polymorphisms were associated with chicken growth and fat traits in an SNP association study [51]. TAG lipolysis is the procedure that decreases energy storage in adipose tissue and may be followed by the increase in fatty acid oxidation, resulting in lean muscle growth, which maximizes feed efficiency and is of critical importance in animal agriculture. Further studies are needed to explore the mechanism in TAG lipolysis, and fatty acid oxidation in chicken adipose tissue. These results indicated a relationship between nutritional status and lipid metabolism and adipogenesis.

Fatty acid oxidation

Fatty acids can be classified as short-chain (SCFAs, C4–C8), medium-chain (MCFAs, C6–C12), long chain fatty acids (LCFA, C10–C16) and very-long-chain fatty acids (VLCFAs: C17–C26) based on their length. SCFAs and MCFAs are oxidized exclusively in the mitochondria. LCFAs long are oxidized in both the mitochondria and the peroxisomes. Fatty acids longer than 14 carbon atoms are preferentially oxidized in the peroxisomes.

Fatty acids destined for oxidation are first activated and converted to fatty acyl-CoA by acyl-CoA synthetases long chain family (ACSL) in the cytoplasm, which is followed by oxidation in the mitochondria. Fatty acyl-CoA is transported into the mitochondria via an acyl-carnitine intermediate, which is generated by carnitine palmitoyltransferase 1 (CPT1), the key enzyme catalyzing mitochondria fatty acid

oxidation. In humans, three isoforms of CPT1 were identified: CPT1A, mostly express in the liver, CPT1B, predominately express in the skeletal muscle, and CPT-1C, exclusively express in the brain and testes. On the inner mitochondria membrane, carnitine palmitoyltransferase 2 (CPT-2) regenerates fatty acyl-CoA molecules from fatty acyl-carnitine.

In contrast to mitochondria, peroxisomes are the site of degradation of VLCFA and the synthesis of the important PUFA (DHA and DPA) by shortening C24:6ω3 and C24:5ω6, respectively [52]. Peroxisome fatty acid oxidation is initiated by the rate-limiting enzyme acyl-CoA oxidases (ACOX). This step yields a significant reactive oxygen species (ROS) by the reduction of O_2 to hydrogen peroxide (H_2O_2). Among three peroxisomal acyl-CoA oxidases, ACOX1 is the most important oxidase that catalyzes the oxidation of VLCFA in humans and rodents.

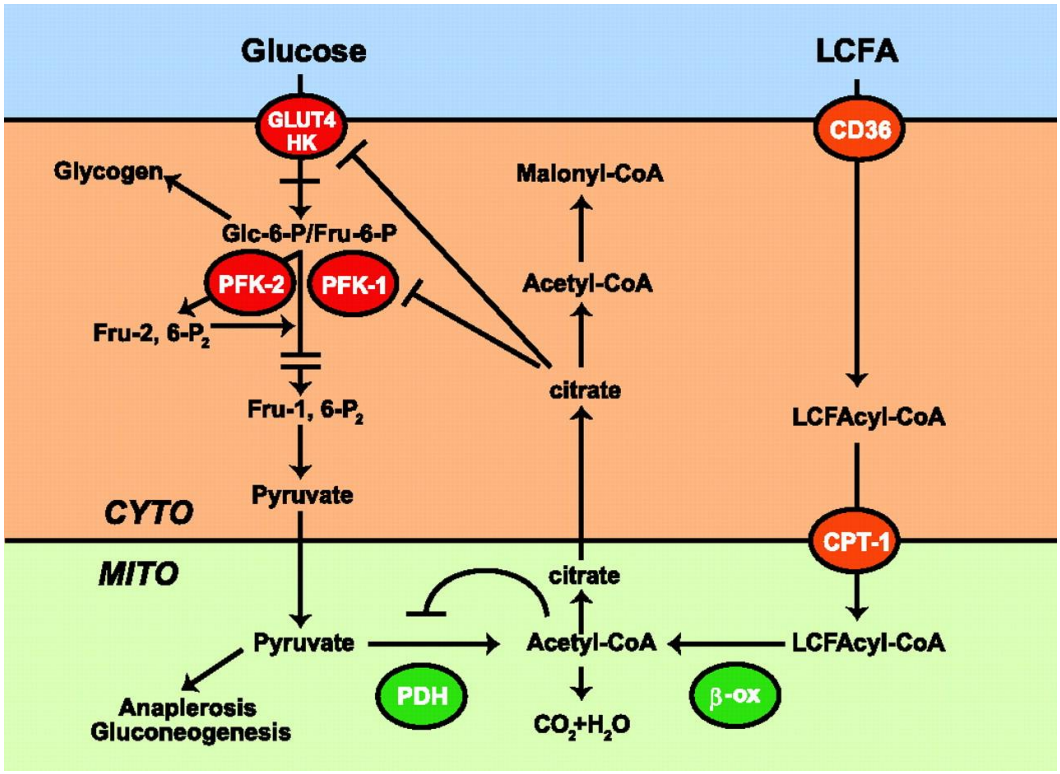


Figure 1-2. Mechanism of inhibition of glucose utilization by fatty acid oxidation. The extent of inhibition is graded and most severe at the level of pyruvate dehydrogenase (PDH) and less severe at the level of 6-phosphofructo-1-kinase (PFK) and glucose uptake. PDH inhibition is caused by acetyl-CoA and NADH accumulation resulting from fatty acid oxidation, whereas PFK inhbition results from citrate accumulation in the cytosol. The mechanism of inhibition of glucose uptake is not clear. These effects reroute glucose toward glycogen synthesis and pyruvate to anaplerosis and/or gluconeogenesis. See text for further details. CYTO, cytosol; MITO, mitochondria , image Image courtesy of Hue, 2009**[53].**

Glucose and fatty acids are the major fuels for metabolism and there is an interaction between their utilization, which is called the glucose-fatty acid cycle.. The glucose-fatty acid cycle, also termed Randle cycle [54], describes reciprocal regulation of carbohydrate and fat metabolism. i.e., elevation of plasma non-esterified fatty acid (NEFA) and fatty acid oxidation inhibit glucose uptake and pyruvate oxidation, and vice versa (Figure 1-2). This phenomenon is prevalent in skeletal muscle and adipose tissue.

As outlined in Figure 1-2, the process is regulated by pyruvate dehydrogenase kinase (PDKs). During fatty acid oxidation, activated PDK phosphorylates and inhibit pyruvate dehydrogenase (PDH), which in turn inhibits pyruvate oxidation. Fatty acid oxidation also leads to elevated NADH, which consequently activate PDKs. Additionally, excess acetyl-CoA serves as the substrate for citrate or acetyl-carnitine in the cytosol. The resultant citrate is an allosteric inhibitor of PFK1 and consequently inhibits glycolysis.

Regulation of Adipose Metabolism

Among the hormones, insulin is studied most often as a regulator of adipose metabolism due to its important roles in glucose and lipid metabolism. In mammals, insulin promotes glucose utilization by stimulating glucose transport and inhibiting lipolysis (Figure 1-3B). The transport of glucose from the plasma membrane to peripheral tissues is mediated by glucose transporter proteins (GLUT), specifically GLUT4, the insulin-responsive glucose transporter highly expressed in muscle and adipose tissue in rodents [55, 56].

Chickens, however, are considered to be refractory to insulin. Chicken is characterized by the unique features of hyperglycemia and insulin resistance. Chickens have a high plasma glucose level of 2 g/l in the fed state under normal insulin

concentration in circulation [57]. This level would be considered diabetic in human. The injection of insulin cause slight hypoglycemia in chickens, but the doses are higher than those required in mammals [57], which indicates the insensitivity of chickens to insulin. However, insulin does play a role in glucose homeostasis in chickens, which was demonstrated by the evidence that immuno-neutralization of insulin largely enhanced plasma glucose in fed broilers [58]. However, the association of glycemia with adiposity of chickens differ from that of mammals in that lower fasting plasma glucose levels are found in fatter chickens [59, 60]. In chicken adipocytes, insulin was found to increase glucose uptake, glucose oxidation, and lipogenesis [61]. However, the effect of insulin on adipocytes was thought to be limited compared to the effect of other hormones, such as glucagon [57].

Not all of the isoforms of glucose transporters in chickens have been identified. The known isoforms expressed in chickens include GLUT1, which is highest expressed in the brain and adipose tissue, GLUT2, which is only expressed in liver and kidney, GLUT3, which is highly expressed in brain, and GLUT8, which is highly expressed in kidney and adipose tissue [55]. Chicks injected with insulin exhibited a significant increase in glucose uptake in cardiac tissue, brain, kidney, and skeletal muscle, but not in cardiac muscle and adipose tissue. This finding suggested that adipose tissue may be excluded from the insulin-responsive glucose transport mechanism [62]. This is not surprising because acetate and pyruvate, rather than glucose, are the major substrates for *de novo* lipogenesis to produce TAG in chickens, and glucose is only the precursor for glyceride-glycerol. Insulin actually affects

lipogenesis by stimulating the incorporation of acetate into lipids, which was observed in isolated chicken adipocytes [63].

To further understand the mechanism of the regulatory roles of insulin in chickens, several chicken models were generated by manipulating diet or insulin deprivation to characterize insulin receptors (IR), as well as other components of the insulin signaling pathways. These studies revealed that different chicken tissues have specific responses to insulin. All of these studies proved that the liver is sensitive to insulinemia in both early steps (IRß, IRS-1, Shc and PI3K) and downstream elements (Akt, MAPK ERK2, GSK3, P70S6K and S6 ribosomal protein) of insulin signaling [64-66]. In contrast, muscle showed insulin resistance, but only in the early steps of insulin signaling [58, 67], whereas adipose tissue was more refractory to insulin than muscles in both the early steps and downstream elements of insulin signaling cascades [68]. Moreover, genetically fat and lean line chickens showed apparent differences in insulin signaling in response to feeding, prolonged fasting and refeeding. Fat line chickens exhibited higher insulin sensitivity in liver than lean line chickens in terms of early steps (IRß, IRS-1, Shc and PI3K) of insulin signaling cascade, which would account for increased liver lipogenesis in fat line chicken [64]. Nevertheless, little difference was observed in leg muscle of fat line chickens regarding the early steps of insulin signaling in response to nutritional status, which may be due to consistent refractory to insulin in muscles [64].

Glucagon is a major lipolytic hormone in chickens [69]. Glucagon binds to receptors on the surface of adipocytes and catalyzes the production of cyclic AMP (cAMP) inside the adipocyte [70]. As in mammals, induction of cAMP mediates the

lipolytic effect of glucagon by activating lipase enzymes. Glucagon-induced cAMP was much higher in chicken adipocyte than in rat adipocyte; this is probably due to the variations in the degradation rate of cAMP [71], which may explain the powerful effect of glucagon in chickens., Other hormones exhibited different effects on chickens. For instance, growth hormone has both lipolytic and anti-lipogenic effects [72]. Further studies are needed to elucidate the effects of thyroid hormones (T3 and T4) and insulin growth factors on adipose physiology and metabolism.

In addition to glucagon, other lipolytic stimuli include catecholamine (i.e., epinephrine, norepinephrine and dopamine), melanocortins (i.e., Adrenocorticotrophin hormone (ACTH) and melanocytestimulating hormone (MSH)), thyroid-stimulating hormone (TSH) and adenosine [73]. The main pathway leading to lipolysis is the cAMP-dependent protein kinase (PKA) pathway (Figure 1-3A). Through this pathway, stimulating G protein coupled receptors (GPCRs) and activating downstream signaling factors like the enzyme adenylyl cyclase lead to increased synthesis of cyclic-AMP (cAMP) from ATP. Increased level of cAMP subsequently activate PKA, resulting in lipolysis [74]. Activation of protein kinase C and stimulation of the mitogen activated protein kinase (MAPK) pathway could also increase lipolytic activity in adipocytes [75]. A recent study found that lipolysis stimulated by β-adrenergic receptor activation, increasing cAMP levels in white adipocytes of rodents, acutely induces mitochondrial uncoupling. This process may also contribute to fatty acid oxidation. The effect was amplified in the absence of which are amplified in the absence of scavenging BSA [76].

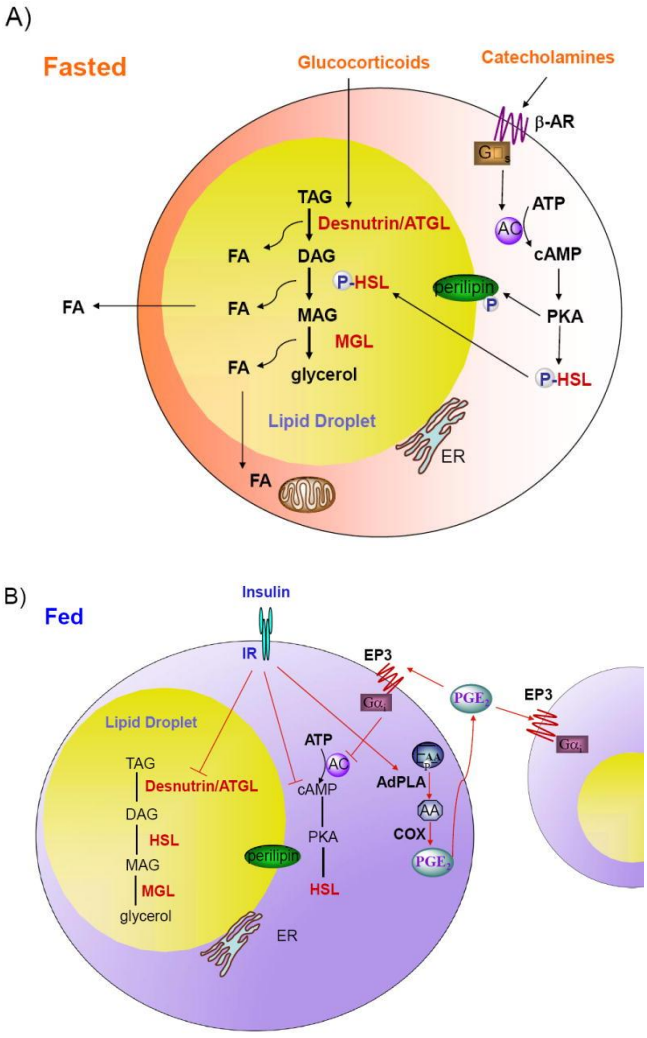


Figure 1-3. Regulation of lipolysis in adipocytes. (A) Desnutrin/ATGL initiates lipolysis by hydrolyzing triacylglycerol (TAG) to diacylglycerol (DAG). During fasting, catecholamines, by binding to Gαs-coupled β-adrenergic receptors (β-AR), activate adenylate cyclase (AC) to increase cAMP and activate protein kinase A (PKA). PKA phosphorylates HSL, resulting in translocation of HSL from the cytosol to the lipid droplet. PKA also phosphorylates the lipid droplet associated protein perilipin. (B) In the fed state, insulin binding to the insulin receptor (IR), results in decreased cAMP levels and decreased lipolysis. Insulin also suppresses expression of desnutrin/ATGL. Image courtesy of Ahmadian, 2010 [74].

Because lipid metabolism pathways and adipose physiology are highly conserved in chickens, hormonal and nutritional regulation of hepatic lipogenesis, adipose fatty acid uptake and the whole process of adipogenesis are comparable between chickens and humans. Understanding the mechanisms of hormonal regulation may also be beneficial in developing new therapies for human obesity.

Mechanisms of Adipose Tissue Expansion

Adipose tissue expands with plasticity to increase TAG uptake and accommodate lipids that would otherwise be deposited ectopically in tissues not intended to store significant amounts of lipid. Adipose tissue can expand its storage capacity by either enlargement of existing adipocytes (hypertrophy) or formation of new adipocytes (hyperplasia). Adipose expansion also requires increasing the local vascular supply, and coordinating different cell types, including endothelial precursor cells, immune cells, and preadipocytes. In humans, adult-onset obesity is associated with hypertrophy, whereas early-onset obesity in children arises from both adipocyte hypertrophy and hyperplasia [27]. The development of hyperplasia is regulated by the growth factors regulating preadipocyte commitment, such as preadipocyte factor-1 (Pref-1, DLK1, SCP-1). Pref1 was found to promote muscle development and inhibit adipose development in mice and play an important role in the regulation of skeletal stem cell differentiation in humans [77].

Adipose cellular development in chicken is characterized by hyperplasia early in life through posthatching to first several weeks of age [78] and it is paralleled by hypertrophy through adulthood. Genetically fat line and lean line chickens show great divergence in both adipocyte size and number, despite a similar feed intake and body

weight [79]. Adipocyte number was two- fold higher in the fat line chickens compared to the lean line chickens at 2 and 5 week of age [23]. Consequently, cell hyperplasia led to high LPL activity in adipose tissue of genetically fat line chickens and enhanced fatty acid uptake in 2-week old fat line chickens [23]. Hypertrophy predominates at older ages, e.g., 12-14 weeks [41]. Therefore, hyperplasia is responsible for early development of fat deposition, which may exacerbate adult obesity. This correlates with the observations in humans that adult-onset obesity is associated with increased adipocyte size, whereas early-onset obesity has both adipocyte hypertrophy and hyperplasia [80]. Adipocyte hypertrophy was considered to be more correlated with excessive fat deposition and more likely to explain the variation of fatness among lines than hyperplasia [81]. Previous studies also illustrated that the difference in plasma triglyceride and phospholipids between fat and lean lines was not significant after nine weeks of age [22], as well as *de novo* fatty acid synthesis in 15-week-old chickens [82], but the difference in abdominal fat was still pronounced. This finding indicated that adipocyte hypertrophy and adipose expansion are probably responsible for the majority of differences in fatness of fat line and lean line chickens during adulthood.

In chicken adipose tissue, full length DLK1 gene also had a myogenic function and had higher expression in preadipocytes than adipocytes in an age dependent manner [83]. Likewise, some growth factors regulate preadipocyte proliferation and differentiation, such as transforming growth factor-ß (TGFß), insulin-like growth factors (IGFs) and fibroblast growth factors (FGF). TGFß also has functions to promote proliferation and inhibit differentiation of adipocytes precursors, which was

also observed in chickens [84, 85]. TGFß also reduces LPL activity in accordance with its anti-lipogenic activity.

A recent study reported the onset of adipocyte hypertrophy as early as five weeks of age in fat line chickens resulting from the sustained selection pressure on the fatness trait. And it also demonstrated that the divergence of adipocyte cell number between the two lines was pronounced from 10 days of age, which indicated ontogenic variation in chicken adipose tissue due to genetic selection [86].

Endocrine Functions of Adipose Tissue

Adipokines

In addition to its classical role as an energy storage depot, white adipose tissue is now recognized as an important endocrine organ due to its ability to synthesize and secret a number of proteins that are collectively referred as adipokines [87]. The term "adipokines" is defined from "adipose cytokine" because many of adipokines are cytokines with known roles in immune functions [88]. Obesity is associated with differentially expression of many adipokines. In general, inflammatory adipkines such as TNFa and IL-6 are at higher levels in obese vs. lean adipose tissues. Adipokines are considered as the molecular link between inflammation, obesity, insulin resistance and other metabolic syndromes in humans [89, 90]. Some adipokines, such as leptin, adiponectin, resistin and visfatin, are involved in energy and glucose homeostasis.

Adipokines exhibit pro-inflammatory and/or anti-inflammatory effects. For example, chemokines such as monocyte chemotactic protein 1 (MCP-1) and interleukin 8 (IL-8), IL-6, IL-1, angiotensin-II (Ang II) and tumor necrosis factor a (TNF-a), have been implicated to be responsible for the development of insulin resistance and the increased risk of cardiovascular disease associated with obesity. By

contrast, induction of anti-inflammatory adipokines, such as IL-10 and adiponectin, inhibit IL-6 and nuclear factor κB (NF-kB) and lead to increased insulin sensitivity and elevated fatty acid oxidation [91].

The relationship between adipokines and obesity has been intensively studied in many other species, including dogs, cats and horses [88], but the relationship in chickens remains unknown. For example, the existence of chicken leptin remains debated [92-94], although leptin receptor (LEPR) has been identified in multiple chicken tissues, including adipose tissue [95-98]. Similarly, TNFa, resistin and omentin, which are extensively studied in mammals, have not been identified in chickens. The expression of adiponectin and visfatin in chicken adipose tissues has been confirmed. Adiponectin is an anti-inflammatory adipokine that enhances insulin sensitivity and is regulated by C/EBPα in humans [99]. Adiponectin gene expression in abdominal adipose tissue in broilers was increased with restricted dietary energy and protein levels, although only at a younger age [100], which was also seen in humans with low-calorie diet [101]. Visfatin was found associated with obesity [12] and type 2 diabetes mellitus [102] through mediating insulin secretion in pancreatic βcells [103]. In chickens, visfatin expression, especially in adipose tissue, was regulated by the factors related to energy homeostasis in a tissue-specific and sex-dependent manner [104]. Therefore, their regulation in adipose tissue of chicken are still of interest.

Adipose tissue expansion is also associated with immune cells infiltration. A number of immune cells reside in adipose tissue, including macrophages, mast cells, B-2 cells, CD8+ T cells, IFN-γ+ CD4+ T cells, eosinophils, and regulatory T cells. They exert

a wide range of functions, which can be detrimental by inducing inflammation and insulin resistance or be beneficial by protecting against these pathologies [28]. These immune cells secrete mediators such as IL-6, TNFa, and MCP1 involved in immune responses. In lean individuals, adipocytes interact with anti-inflammatory immune cells, such as eosinophils and regulatory T-lymphocytes (Treg). However, in obese individuals, obesity drives the interaction with inflammatory immune cells and stimulates the infiltration macrophages (Figure 1-4).

Macrophages that infiltrate adipose tissue contribute to the increased expression of inflammatory cytokines in obesity in human and rodents [105]. Adipose tissue macrophages are classified into M1 phenotype which secretes high level of pro-inflammatory cytokines, such as IL-6 and TNF-α, and M2 phenotype which mainly secretes anti-inflammatory cytokines, such as IL-10. Adipose tissue from obese animals give rise to the M1 phenotype by expressing high levels of chemokines, i.e., MCP-1, or stimulated by TLR4 ligands, i.e., saturated fatty acids. In contrast, macrophages maintain the M2 phenotype in lean adipose tissue [105]. Little is known about the presence, recruitment or types of macrophages in chicken adipose tissue. It was found that lipogenesis in chicken adipocytes, rather than hepatocytes, was increased by conditioned medium from chicken macrophages [106], which suggest a role for macrophage infiltration in chicken adipose accumulation.

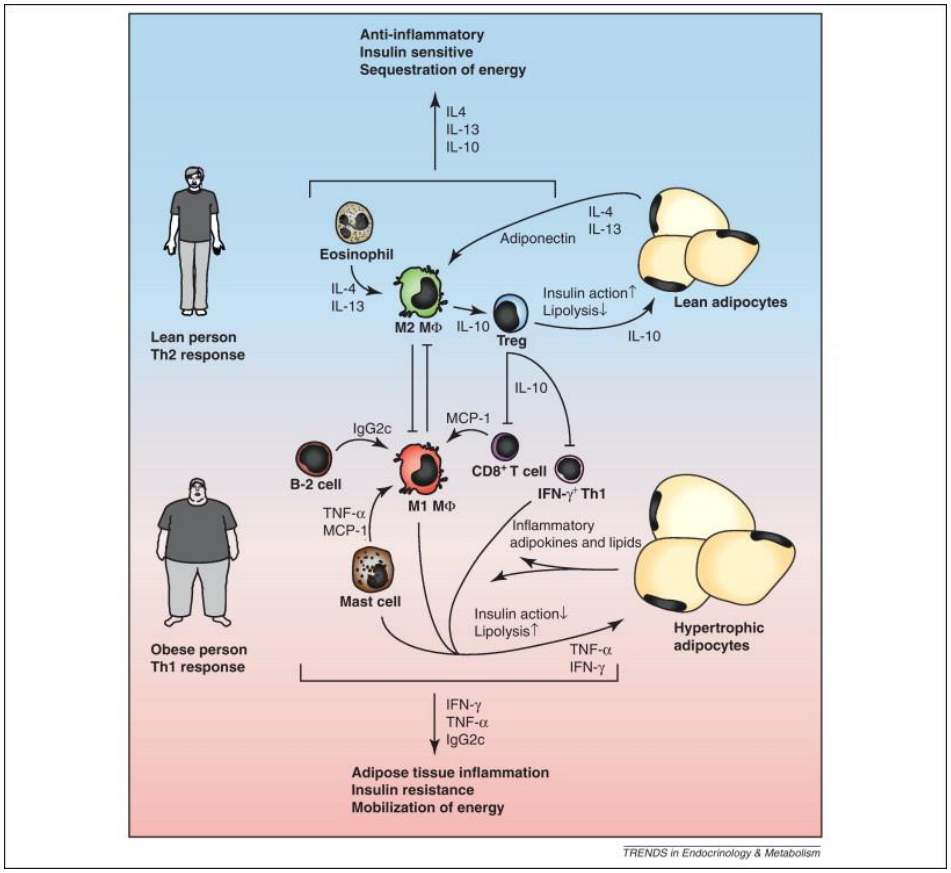


Figure 1-4. Schematic representation of adipocyte–immune-cell interactions and the soluble mediators involved in adipose tissue of lean versus obese individuals. Image courtesy of Schipper, 2012 [28].

Genetic Mapping of Fatness-synteny with Human Genetic Studies of Obesity

For decades, quantative trait loci (QTL) have been used to identify the genes that affect disease resistance and productivity using different types of genetic crosses. For this reason, a dense linkage map has been produced to enrich genetic resources. The availability of genomic and genetic resources, including the genome sequence, the high-density linkage map, and the newly developed web-based tool Chicken Obesity Gene Map in Gallus Gbrowse, enable large scale analyses of gene function using chickens as an animal model for human research. Linkage mapping suggest that the chicken genome has a better synteny with the human genome than the mouse genome; there are around 100 conserved synergic groups between chicken and human, which in mouse and human are 167 [107].

Chicken F2 populations generated from chickens divergently selected for low or high growth rate and abdominal fatness provide valuable models through which to integrate phenotypes with genotypes. Genome-wide linkage analyses on F2 populations have identified QTLs associated with body composition, growth and metabolic traits [108-110]. Additionally, F2 populations generated from fast-growing broilers male line and 2 slow-growing inbred lines (Leghorn and Fayoumi) of chicken populations provide comparative models to study growth and fat deposition in chickens. Genome-wide studies on these F2 chickens have identified QTLs associated with abdominal fat percentage, growth and average daily gain, body composition, skeletal integrity and metabolic traits in the chickens [111-114].

These studies found that adiposity in chickens is polygenic and under the control of multiple chromosomal loci, which have high synteny with human obesity. Most of the genes located on QTL regions for the fatness trait were found to be obesity and diabetes related genes in humans [115-117]. For example, QTL mapping of metabolic and body composition traits in F2 intercross between high-growth and low-growth chicken lines revealed that QTL for fatness and plasma glucose are co-localized on several loci, which control each trait in the same direction [115]. This finding fits with the metabolic deviations observed in obese humans with hyperglycemia [115]. Furthermore, GLUT12 gene, which was found associated with hypertension and diabetic nephropathy in mammals, was targeted for plasma glucose level by QTL analysis. Additionally, PPARγ, one example of the common genes mapped with human obesity, was identified to be associated with plasma glucose and NEFA in this study [115, 117]. Such comparative studies provide unique opportunities for fully exploring the genetic causes of obesity and T2D in humans.

CHAPTER II

TRANSCRIPTOMIC AND METABOLOMIC PROFILING OF CHICKEN ADIPOSE TISSUE IN RESPONSE TO INSULIN NEUTRALIZATION AND FASTING

This chapter is adapted from the following publication:

Bo Ji, Ben Ernest, Jessica R Gooding, Suchita Das, Arnold M Saxton, Jean Simon, Joelle Dupont, Sonia M étayer-Coustard, Shawn R Campagna, and Brynn H Voy.
BMC Genomics 2012 13:441.

Abstract

Domestic broiler chickens rapidly accumulate adipose tissue due to intensive genetic selection for rapid growth and are naturally hyperglycemic and insulin resistant, making them an attractive addition to the suite of rodent models used for studies of obesity and type 2 diabetes in humans. Furthermore, chicken adipose tissue is considered as poorly sensitive to insulin and lipolysis is under glucagon control. Excessive fat accumulation is also an economic and environmental concern for the broiler industry due to the loss of feed efficiency and excessive nitrogen wasting, as well as a negative trait for consumers who are increasingly conscious of dietary fat intake. Understanding the control of avian adipose tissue metabolism would both enhance the utility of chicken as a model organism for human obesity and insulin resistance and highlight new approaches to reduce fat deposition in commercial chickens. We combined transcriptomics and metabolomics to characterize the response of chicken adipose tissue to two energy manipulations, fasting and insulin deprivation in the fed state. Sixteen to 17 day-old

commercial broiler chickens (ISA915) were fed *ad libitum*, fasted for five hours, or fed but deprived of insulin by injections of anti-insulin serum. Pair-wise contrasts of expression data identified a total of 2016 genes that were differentially expressed after correction for multiple testing, with the vast majority of differences due to fasting (1780 genes). Gene Ontology and KEGG pathway analyses indicated that a short term fast impacted expression of genes in a broad selection of pathways related to metabolism, signaling and adipogenesis. The effects of insulin neutralization largely overlapped with the response to fasting, but with more modest effects on adipose tissue metabolism. Tissue metabolomics indicated unique effects of insulin on amino acid metabolism. Collectively, these data provide a foundation for further study into the molecular basis for adipose expansion in commercial poultry and identify potential pathways through which fat accretion may be attenuated in the future through genetic selection or management practices. They also highlight chicken as a useful model organism in which to study the dynamic relationship between food intake, metabolism, and adipose tissue biology

Introduction

The domestic chicken provides a widespread and relatively inexpensive source of dietary protein for humans. In addition to its role as a food animal, the chicken has a long history as a valuable model research organism [118]. These dual considerations led to the selection of chicken as the first agricultural animal model to be sequenced at the genome level [119]. While chickens have been used heavily for studies of developmental biology and immunology, a number of traits make them a viable model for studies of adipose biology, obesity and insulin resistance. Commercial broiler chickens, in particular,

rapidly accumulate excess adipose tissue as a result of genetic selection for growth and are considered "obese" relative to leaner egg-laying or wild strains of chickens (rev. in [120]). Chickens mimic the early stage of type 2 diabetes in humans, exhibiting both hyperglycemia (up to 200 mg/dL in the fasting state) and resistance to exogenous insulin [121, 122]. Like humans, but unlike rodents or pigs, chickens rely on liver rather than adipose tissue for the majority of *de novo* lipid synthesis [123-125]. Most metabolic genes are conserved with humans, and a number of the quantitative trait loci (QTLs) that have been linked to fatness in chickens contain genes implicated in human susceptibility to obesity or diabetes [126]. Chickens also represent a model for studying mechanisms of adipocyte hyperplasia during development, a process that may exacerbate adult obesity. During at least the first several weeks after hatch, chicken adipose tissue expands more through adipocyte hyperplasia than hypertrophy, and an early increase in adipocyte number is a common feature of some lines genetically selected for excess adiposity [11, 127]. Finally, the egg presents opportunities to directly manipulate the developmental milieu and study the consequences on adipose metabolism via *in ovo* injection.

Relatively little is known about regulation of adipose tissue deposition and metabolism in chicken. Because of its relative importance in lipogenesis, most studies have focused on the role of liver in adipose expansion. Several genetic lines of fat and lean chickens have been developed through phenotypic selection, most of which have both elevated plasma levels of VLDL and lower levels of plasma glucose, reflecting the importance of hepatic lipogenesis and glucose consumption in fat accretion. Reciprocally, phenotypic selection for low plasma glucose simultaneously selects for fatness [128]. Both chicken and mammalian adipocytes develop through a sequence of molecular

triggers including activation of CEBPα and PPARγ [129]. A clear point of divergence, however, is their responsiveness to insulin. Unlike in mammals, insulin has minimal effect on glucose uptake in chicken adipose tissue [62]. In fact, an avian homolog of the insulin-sensitive glucose transporter GLUT4 has not been identified in the current chicken genome database. Insulin does, however, stimulate uptake of acetate, which is the preferred substrate for *de novo* lipogenesis in chicken adipocytes, although the magnitude of the effect is relatively modest [130]. Insulin signaling appears to proceed through tissue specific cascades in chicken metabolic tissues. In liver, insulin elicits a signaling cascade that parallels the response in mammals, including tyrosine phosphorylation of insulin receptor β-subunit (IRβ), insulin receptor substrate-1 (IRS-1) and Src homology 2 domain-containing substrate (Shc) and activation of phosphatidylinositol 3-kinase (PI3K) [65, 131]. The situation in skeletal muscle is more complex. Tyrosine phosphorylation of IRβ and IRS-1 and PI3K activity are not regulated by insulin, whereas events downstream of PI3K (e.g. Akt and P70S6K activation) are accordingly sensitive [58]. We recently reported that insulin also does not elicit a classical IRβinitiated cascade in chicken adipose tissue, including the downstream steps of Akt and P70S6K activation [132]. Insulin also does not inhibit lipolysis in chicken adipose tissue; glucagon, is the primary lipolytic hormone (rev. in [133]).

In the present study we simultaneously characterized the effects of a short term (5 hours) fast or neutralization of insulin action (5 hours) on adipose tissue of young (16–17 day-old), fed commercial broiler chickens. The goals of this study were two-fold. First, we sought to identify pathways activated by feed restriction, reasoning that they may highlight potential strategies for control of fatness through either genetic selection or

improved management practices. Simultaneously, we sought to understand the contribution of insulin, if any, into chicken adipose physiology. No experimental model of diabetes exist in chicken: total pancreatectomies are not achievable, and alloxan and streptozotocin are inefficient at destroying pancreatic chicken beta-cells (rev. in [122]). The two treatments were compared to distinguish potential insulin-specific changes from those that could be mimicked by fasting through changes in nutrient availability. Both treatments were shown previously to elicit significant alterations in several plasma metabolic and endocrine parameters [58]; in the studies reported herein, samples of abdominal adipose tissue were issued from the same experiment. Tissue metabolomics was combined with microarrays to bridge the gap between gene expression, metabolic and physiological responses, and to identify the composite effects of both fasting and insulin deprivation on chicken adipose tissue.

Materials and Methods

Animals

Male broiler chicks (ISA 915, Institut de Se lection Animale, Saint Brieuc, France) from which samples were collected for this study were hatched and raised under standard conditions, as originally described by Dupont [58] and in accordance with the guidelines for Care and Use of Agricultural Animals in Agricultural Research and Teaching. Briefly, at 16–17 days of age, chicks of similar body weights were either allowed to continue feeding (*ad libitum* fed controls), fasted for five hours, or fed but injected at 0, 2 and 4 hours with porcine anti-insulin serum (insulin neutralized). Both the fed and fasted groups received injections of normal porcine serum as a vehicle control. Abdominal

adipose tissue samples were harvested and rapidly snap-frozen in liquid nitrogen, pulverized, then stored at -80 ℃ until analysis. Adipose samples from five birds in each group were used for both microarray and metabolomic analyses.

Gene expression

Total RNA was isolated from chicken adipose samples using the RNeasy Lipid kit and incorporating an on-column DNase treated with the RNase-free DNase Set according to the manufacturer's protocol (Qiagen.com). RNA quality and concentration were measured using the Experion System (Bio-Rad.com); only RNA passing recommended standards of quality was used for further studies. Transcriptome profiling was performed by Genome Quebec (Montreal, Canada) using the Affymetrix GeneChip Chicken Genome Array (San Diego, CA). Microarray data from this study are deposited in the Gene Expression Omnibus (GEO) under the accession number GSE35581. For real time RT-PCR validation, cDNA was synthesized using the iScript cDNA Synthesis kit (Bio-Rad.com). Commercially designed and validated primer sets (QuantiTect) and the associated SYBR Green master mix (Qiagen.com) were used to assay gene expression on a CFX96 real-time PCR detection system (Bio-Rad.com). All samples were analyzed in triplicate and normalized to ß-tubulin. Relative differences in gene expression were determined using the $2^{-\Delta\Delta CT}$ method and statistical differences were tested by analysis of variance (ANOVA) [134].

LC-MS/MS

Abdominal adipose tissue samples from five birds in each treatment group (the same five birds used for expression profiling) were extracted by placing tissue in a mortar containing liquid nitrogen and then powdering with a pestle. Portions (8–40 mg) of the powered tissue were weighed into 1.5 mL centrifuge tubes. Chilled methanol (0.5 mL at −80°C) and internal standard (5 μL of 1.7 mM benzoic acid in negative mode or 4.25 mM tris(hydroxymethyl)aminomethane in positive mode) were added to each tube. Each tube was mixed thoroughly by vortexing for two minutes, and the metabolites were extracted from the tissue for 30 min at 4 ℃. The tubes were then centrifuged (5 min, 4 ℃, 16.1 rcf) and supernatant (210 μL) was split into two autosampler vials. One of these samples was immediately placed on the LC-MS/MS for analysis, while the other was stored at −80°C for analysis in the opposite polarity ion mode on the following day.

Samples were placed in an autosampler tray chilled to 4°C, and 10 μL of each was injected onto an LC column for analysis. The chromatography method for positive ion mode was reported previously by Bajad and coworkers [135], with one exception that the column was cooled to 10 ℃. The chromatography method for negative ion mode was performed as reported by Waters and coworkers [136], except the gradient was allowed to run 50 min instead of 45 min to allow more thorough equilibration of the column. The eluent was introduced directly into the MS via an electrospray ionization (ESI) source fitted to a Finnigan TSQ Quantum Discovery Max triple quadrupole MS (Thermo Electron, Waltham, MA) through a 0.1 mm internal diameter fused silica capillary. The spray voltage was 4500 V in positive mode or 3000 V in negative mode. The sheath gas

(nitrogen) was set to 40 psi, and the capillary temperature was set to 290 ℃. The collision cell gas (argon) was set to a pressure of 1.5 mTorr. Samples were analyzed using selected reaction monitoring (SRM) mode with a scan width of 1 *m/z* and a scan time of 0.05 s. The SRM parameters for most metabolites have been published previously [135]. This method was used to scan for almost 300 metabolites. Xcalibur software (Thermo Scientific, Waltham, MA) was used to manually assess the elution time of the correct LC spectral peak for each metabolite-specific SRM. The Quan Browser utility in Xcalibur was then used to integrate the LC spectral peak area (in ion counts) for each detected compound, and these data were exported to a Microsoft Excel spreadsheet for further processing.

Statistical analysis

Statistical analysis of the microarray data was performed using R 2.9.0 and routines contained in Bioconductor (bioconductor.org). GC robust multi-array average (GCRMA) was used to normalize and scale the raw data from CEL files. The normalized data were filtered for low expression by removing any probes with normalized expression less than 3 in at least 5 arrays. Statistical significance of gene expression differences were analyzed by one-way ANOVA and empirical bayes using the limma package. Differential expression was defined based on false discovery rate (FDR) adjusted p-value <0.05 [137]. Venn diagrams of differentially expressed genes were plotted to visualize the number of differentially expressed genes for each treatment comparison and their intersections. Hierarchical clustering of significant genes was performed using the hclust function and a hierarchical clustering heatmap was created using heatmap.2 in the gplots package.

Hierarchical clustering also was used to identify correlated patterns of gene expression and metabolites. The Database for Annotation, Visualization and Integrated Discovery (DAVID, version 6.7) and ClueGO, a Cytoscape plug-in, were used for Gene Ontology (GO) at level 6 and 7 and KEGG analysis of differentially expressed genes [138, 139]. Statistical analysis of metabolomic data was performed using an analysis tool that we developed specifically for metabolomic data analyses [140]. The script (metabR), written in the language R, uses linear mixed-effect modeling to normalize metabolomics data containing both fixed- and random-effect confounding variables. The script averages any replicate measurements (statistical sampling) made on experimental units and performs ANOVA to test for statistical differences between experimental groups.

Results

Expression levels of a total of 2016 genes were significantly altered by fasting and/or insulin neutralization when compared to fed controls based on an FDR adjusted p-value < 0.05 (Additional file 1; Figure 2-1A). Sixty-nine percent of these genes showed a fold-change $\geq |1.5|$ (Figure 2-1B). The majority of changes in expression were attributable to fasting, with 917 up-regulated and 863 down-regulated genes in fasted vs. fed adipose tissue. Insulin neutralization altered expression of 92 genes, 72 of which were also differentially expressed with fasting (Figure 2-1A). All genes that were affected by both treatments changed in the same direction (i.e., up- or down-regulated in both groups). Real-time RT-PCR was employed to validate differential expression based on the microarray data. Eleven genes were selected based on fold-change or biological functions of interest (Table 2-1). Differential expression under fasting versus fed conditions was

validated for all genes except pre-B-cell leukemia homeobox 3 (PBX3). Ten of the eleven genes were also differentially expressed in insulin neutralized compared to fed birds based on QPCR.

Genes that were differentially expressed in at least one pairwise comparison were clustered to visualize the similarities between groups and to determine if insulin-neutralized expression profiles were more similar to fasted or to fed status. As shown in Figure 2-2A, samples within each of the three experimental groups clustered together. The dendrogram also showed that the fasting group was distant from fed and insulin-neutralized groups, which were closer to each other. To further visualize relationships between treatments with regard to gene expression, distinct clusters of genes were extracted and submitted to gene set enrichment analysis to identify GO terms and pathways that were significantly overrepresented among genes contained in these clusters. Seven clusters represented four general patterns of similarities between treatments (Figure 2-2B). Clusters 1, 3 and 4 consisted of genes with higher expression in fasting compared to both insulin-neutralized and fed conditions, with insulin-neutralized intermediate between fasted and fed. This set of genes was significantly enriched in GO terms related to protein and lipid catabolism and to cell signaling, including regulation of the stress-sensitive NFκB cascade (Table 2-2). These three clusters were also enriched in members of the KEGG pathways ubiquitin mediated proteolysis, sphingolipid metabolism, PPAR signaling, fatty acid metabolism and the peroxisome. The rate-limiting genes for fatty acid oxidation (ACOX1 and CPT1A), along with fatty acid binding proteins 5 and 6, are contained in these three clusters. Clusters 5 and 7 also contained genes with higher levels in fasted vs. the other two groups, but with

comparable expression levels between insulin-neutralized and fed, and thus no clear effect of insulin loss.

These two clusters were significantly enriched in pathways related to signaling and metabolism, including enzyme linked receptor protein signaling pathway ($p = 0.0097$) and in the KEGG pathways for glycerolipid metabolism and PPAR. Genes responsible for the latter enrichment include PPARΔ, which was recently shown to increase total oxidative metabolism in white adipose tissue [141]. Clusters 2 and 6 contained genes expressed at lowest levels in fasted chickens. Genes in cluster 2 were expressed at intermediate levels in the insulin-neutralized group relative to fed and fasted. This set of genes was significantly enriched in GO annotations related to monosaccharide catabolic process and glucose metabolism, and in genes comprising the KEGG pathways for carbohydrate metabolism, TCA cycle and glycolysis (Table 3-2). Finally, cluster 6 consisted of genes that were also lowest in fasting but showed no clear effect of insulin loss, with similar expression in fed and insulin-neutralized groups. This set of genes was significantly enriched for the KEGG pathways steroid biosynthesis ($p = 0.0043$), glyoxylate and dicarboxylate metabolism ($P = 0.012$) and pyruvate metabolism ($P = 0.033$), along with a number of genes involved in lipid biosynthesis, which was the highest scoring GO category ($p = 0.6$). Cluster 8 was a distinct, small cluster with variable expression within group and no significant GO or KEGG annotations.

Global biological responses to fasting and to insulin neutralization were further characterized using KEGG pathway matching, based on genes with statistically significant differential expression ($FDR < 0.05$) and absolute fold-change ≥ 1.5. Genes

altered exclusively by fasting represented a wide range of cellular pathways, indicating significant effects of even a five hour fast on adipose function and metabolism in chicken. Fasting exerted significant effects on pathways related to carbohydrate, amino acid and lipid metabolism and synthesis. Within the categories related to lipid metabolism, fasting up-regulated expression of genes involved in fatty acid oxidation (e.g., acyl-CoA oxidase 1 (ACOX1), acetyl-CoA carboxylase beta (ACACB), carnitine palmitoyltransferase 1A (CPT1A)) and down-regulated expression of genes that control fatty acid, cholesterol and triacylglycerol synthesis (e.g., 1-acylglycerol-3-phosphate O-acyltransferase 9 (AGPAT9), ATP citrate lyase (ACLY), farnesyl diphosphate synthase (FDPS), acetyl-Coenzyme A carboxylase alpha (ACACA) and acetoacetyl-CoA synthetase (AACS)). Fasting also up-regulated expression of many genes involved in proteolysis and amino acid degradation. In addition to pathways highlighted by KEGG analysis, fasting down-regulated a number of genes (e.g., TGFβ, BMP) that mediate mesenchymal stem cell (MSC) commitment, an early step in the formation of new adipocytes (Additional file 1). Finally, a number of phosphodiesterases were up-regulated with fasting, presumably in response to the increased plasma glucagon [58] and subsequent elevations in cyclic adenosine monophosphate (cAMP; Additional file 1). Collectively, these categories indicate that chicken adipose tissue responds to a relatively short duration (five hour) fast with sweeping changes in gene expression that suppress synthesis and storage of lipids and other macromolecules and up-regulate mobilization and metabolism of fatty acids and proteins.

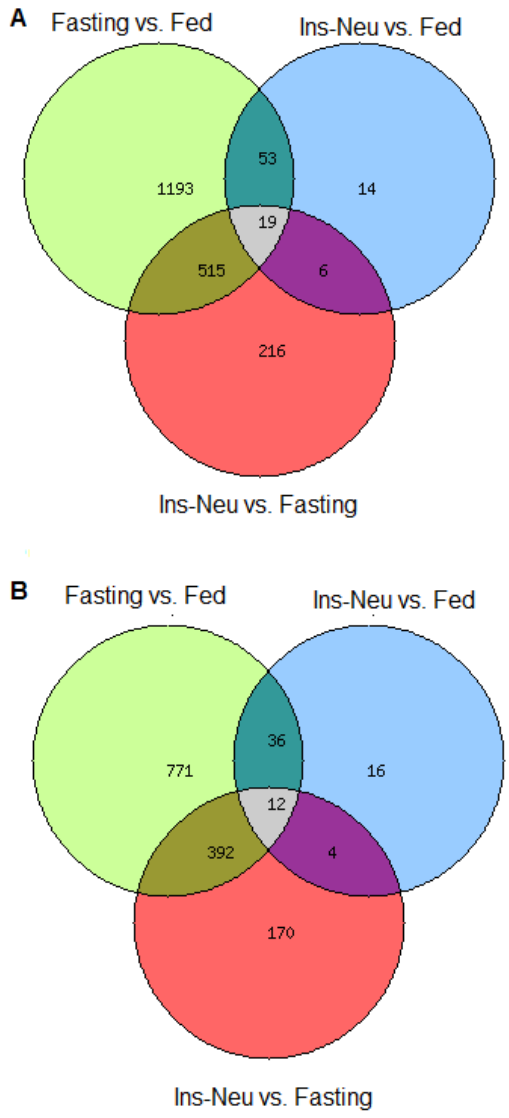


Figure 2-1 Venn diagram of overlapping and unique effects of fasting and insulin neutralization on gene expression. (A) A total of 2016 unique genes were differentially expressed (FDR adjusted p-value <0.05) between one or more pairwise treatment comparisons; (B) A total of 1401 genes with absolute fold change ≥1.5 among the differentially expressed genes

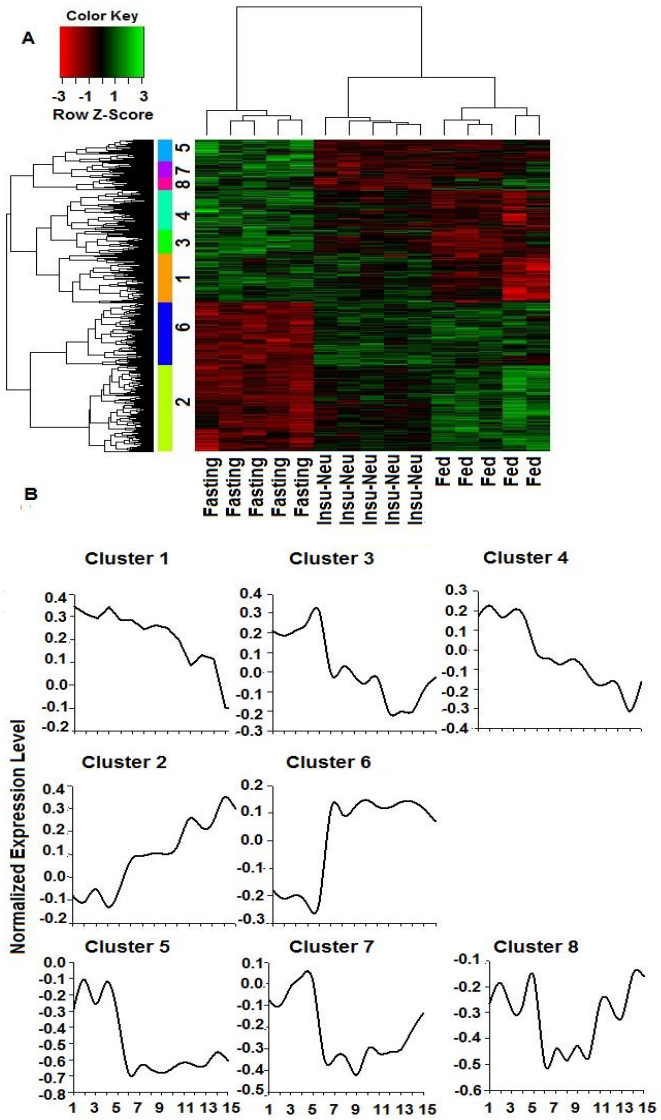


Figure 2-2 Cluster analysis of differentially expressed genes. (A). Hierarchical cluster analysis of the 2016 genes (FDR adjusted p-value <0.05) that were differentially expressed between insulin-neutralized vs. fed and/or fasted vs. fed states. (B) Seven clusters (numbers) representing the most distinct effects of treatment were selected to further analyze expression profiles across treatments. Sample ID number on the X-axis corresponds to treatment group: sample 1–5, fasted; 6–10, insulin neutralized; 11–15,fed control. Y-axis represents relative gene expression value

Table 2-1 Fold change verification of gene expression by RT-PCR

		Microarray Fold Change			Quantitative PCR Fold Change		
Gene Symbol	**Gene Title**	**Fasting vs. Fed**	**Ins-Neu vs. Fed**	**Ins-Neu vs. Fasting**	**Fasting vs. Fed**	**Ins-Neu vs. Fed**	**Ins-Neu vs. Fasting**
FBXO8	F-box protein 8	4.16***	2.09**	-1.99***	2.59***	2.76***	1.06
DUSP5	Dual specificity phosphatase 5	9.43***	1.05	-8.98***	8.13***	1.69***	-4.82***
BNIP3	BCL2/adenovirus E1B 19kDa interacting protein 3	2.98***	1.53	-1.95**	3.45***	2.26***	-1.52*
PBX3	Pre-B-cell leukemia homeobox 3	-1.62***	1.07	1.75***	-1.22	1.83***	2.22***
IL10RB	Interleukin 10 receptor, beta	1.74***	1.02	-1.71**	1.76***	1.42*	-1.24
EGR1	Early growth response 1	2.43*	-1.58	-3.86**	2.58***	-1.36	-3.52***
NAB1	NGFI-A binding protein 1 (EGR1 binding protein 1)	2.43***	1.06	-2.29***	1.67**	1.52**	-1.10
PDK4	Pyruvate dehydrogenase kinase, isozyme 4	17.28***	7.06**	-2.45**	18.33***	4.01***	-4.57***
CTSL2	Cathepsin L2	2.09***	1.55*	-1.35*	2.96***	2.053***	-1.44
AGTR1	Angiotensin II receptor, type 1	4.15 ***	2.05*	-2.02**	3.52**	1.72*	-2.05*
SESN1	Sestrin 1	1.86**	1.125	-1.65	1.58*	1.47*	1.07

FDR p-value: * $p < 0.05$, ** $p < 0.005$, *** $p < 0.0005$

Table 2-2 Gene ontology (GO) and KEGG annotation for representative clusters of differentially expressed genes

Cluster	Annotation	GO term (Biological Process, level 6 or 7) or KEGG pathway name	FDR p-value
1, 3 and 4 (731 genes)	**GO**	Positive regulation of protein metabolic process	6.0 E-3
		Negative regulation of cellular macromolecule biosynthetic process	7.3 E-3
		Triglyceride metabolic process	1.0 E-2
		Negative regulation of gene expression	1.0 E-2
		Proteolysis involved in cellular protein catabolic process	1.0 E-2
		Negative regulation of kinase activity	1.5 E-2
		Regulation of transcription, DNA-dependent	2.1 E-2
		Protein phosphorylation	3.3 E-2
		Antigen receptor-mediated signaling pathway	3.3 E-2
		Regulation of phosphate metabolic process	3.4 E-2
		Regulation of kinase activity	3.4 E-2
		Regulation of I-kappaB kinase/NF-kappaB cascade	3.5 E-2
	KEGG	Ubiquitin mediated proteolysis	1.0 E-2
		Sphingolipid metabolism	1.8 E-2
		PPAR signaling pathway	2.4 E-2
		Fatty acid metabolism	4.6 E-2
		Peroxisome	5.0 E-2
2 (557 genes)	**GO**	Monosaccharide catabolic process	2.5 E-2
		DNA dependent DNA replication	3.6 E-3
		Hexose metabolic process	1.1 E-2
		Glucose metabolic process	1.4 E-2
		Regulation of cell shape	1.5 E-2
		DNA replication	1.5 E-2
		Nucleoside triphosphate metabolic	2.3 E-2
	KEGG	Glycolysis / Gluconeogenesis	4.9 E-4
		Citrate cycle (TCA cycle)	8.9 E-4
6 (402 genes)	**KEGG**	Steroid biosynthesis	4.4 E-3
		Glyoxylate and dicarboxylate metabolism	1.2 E-2
		Pyruvate metabolism	3.3 E-2
5 and 7 (250 genes)	**GO**	Enzyme linked receptor protein signaling pathway	9.7 E-3
		Regulation of cellular protein metabolic process	3.1 E-2
		Negative regulation of macromolecule metabolic process	3.5 E-2
		Transmembrane receptor protein serine/threonine kinase signaling pathway	3.6 E-2
	KEGG	PPAR signaling pathway	1.0 E-4
		Glycerolipid metabolism	1.8 E-2
		MAPK signaling pathway	4.6 E-2

Loss of insulin action also resulted in significant effects on adipose gene expression, the majority of which overlapped with the response to fasting (Table 2-3; Additional file 2). Several genes central to energy metabolism were affected. Diacylglycerol O-acyltransferase homolog 2 (DGAT2), which catalyzes the final and only committed step in tracylglycerol synthesis, was down-regulated (10.5-and 6.1-fold, respectively, fasted and insulin neutralized) in both treatment groups relative to the fed group. Conversely, acyl-Coenzyme A binding domain containing 5 (ACBD5) and pyruvate dehydrogenase kinase 4 (PDK4) were significantly up-regulated in both treatments relative to fed controls. ACBD5 is one of a family of long chain fatty acyl CoA trafficking protein proteins that play roles in both triglyceride synthesis and beta-oxidation [142]. PDK4, which was up-regulated vs. fed by ~ 17-fold with fasting and 6-fold with insulin neutralization, acts as a fuel switch by phosphorylating and inactivating pyruvate dehydrogenase, shifting metabolism from glycolysis to fatty acid oxidation [143]. Fasting and insulin neutralization also up-regulated expression of the type I angiotensin II receptor (AGTR1). Angiotensin II alters adipocyte lipid metabolism and insulin signaling [144-146], and increased AGTR1 expression in adipose tissue is associated with enhanced insulin sensitivity [147]. Finally, a number of genes regulated by both fasting and insulin neutralization function in general processes related to protein synthesis.

A total of thirteen genes (four up-regulated and nine down-regulated) were differentially expressed only with insulin neutralization (Table 2-4). The most interesting of these responses were upregulation of GCG, which encodes preproglucagon (fold change = 2.91), in parallel with downregulation of the glucagon receptor (LOC425670,

fold change = −2.77). Other genes uniquely affected by insulin have less clear relevance to adipose biology according to current knowledge.

Table 2-3 Shared effects of fasting and insulin-neutralization on differential gene expression

Gene Symbol	Gene Title	Fold change Insu-Neu vs. Fed	Fold change Fasting vs. Fed
Up-regulated genes			
PDK4	pyruvate dehydrogenase kinase, isozyme 4	7.06	17.28
AOX1	aldehyde oxidase 1	2.94	6.66
PLEKHH2	pleckstrin homology domain containing, family H (with MyTH4 domain) member 2	2.41	2.77
FBXO8	F-box protein 8	2.09	4.15
AGTR1	angiotensin II receptor, type 1	2.04	4.15
PCMTD1	protein-L-isoaspartate (D-aspartate) O-methyltransferase domain containing 1	1.99	2.25
PSME4	proteasome (prosome, macropain) activator subunit 4	1.97	3.86
ICA1	islet cell autoantigen 1, 69kDa	1.85	2.8
IP6K2	inositol hexakisphosphate kinase 2	1.77	2.75
UHRF2	ubiquitin-like, containing PHD and RING finger domains, 2	1.72	1.89
ACBD5	acyl-Coenzyme A binding domain containing 5	1.72	2.51
LOC417776	similar to hypothetical protein	1.62	1.84
CTSL2	cathepsin L2	1.54	2.09
ZNF217	zinc finger protein 217	1.54	2.6
IFNAR1	Interferon (alpha, beta and omega) receptor 1	1.52	3.47
Down-regulated genes			
DGAT2	diacylglycerol O-acyltransferase homolog 2 (mouse)	6.1	10.5
EEPD1	endonuclease/exonuclease/phosphatase family domain containing 1	2.4	2.17
ANKRD9	ankyrin repeat domain 9	2.19	1.98

Table 2-3 Continued

Gene Symbol	Gene Title	Fold change Insu-Neu vs. Fed	Fold change Fasting vs. Fed
Down-regulated genes			
DLST	dihydrolipoamide S-succinyltransferase (E2 component of 2-oxo-glutarate complex)	1.95	1.76
PTP4A3	protein tyrosine phosphatase type IVA, member 3	1.8	2.3
HSPA5	heat shock 70kDa protein 5 (glucose-regulated protein, 78kDa)	1.76	1.74
NOLA2	nucleolar protein family A, member 2 (H/ACA small nucleolar RNPs)	1.73	1.57
MST1R	macrophage stimulating 1 receptor (c-met-related tyrosine kinase)	1.71	2.2
GRAMD2	GRAM domain containing 2	1.69	2.37
DHDDS	dehydrodolichyl diphosphate synthase	1.63	1.58
DYNLL2	dynein, light chain, LC8-type 2	1.6	2.52
CDT1	chromatin licensing and DNA replication factor 1	1.57	1.51
FAHD1	fumarylacetoacetate hydrolase domain containing 1	1.56	1.61
DOT1L	DOT1-like, histone H3 methyltransferase (S. cerevisiae)	1.56	1.82
BTBD11	BTB (POZ) domain containing 11	1.55	1.71

FDR p-value <0.05

Tissue metabolomic analysis was used to identify the metabolic intermediates that were altered by fasting and insulin neutralization. A total of 92 metabolites were detected based on signal-to-noise ratios (Additional file 3). It is worth noting that glucose-6-phosphate content was similar in fasted or "diabetic" *vs.* fed status, despite a large range of plasma glucose levels (232–747 mg/100 ml). A total of 12 metabolites were significantly different between treatment groups based on $p < 0.05$ and an additional five were suggestive of significance ($p < 0.10$; Table 2-5). Tissue levels of amino acids were consistently lower in fasted vs. fed tissue, with statistically significant reductions in asparagine and glutamine ($p < 0.01$). Presumably, these effects were due to a change in the balance of protein synthesis/proteolysis and to the catabolism of carbon skeletons for energy in response to energy restriction, which is consistent with up-regulated expression of genes involved in amino acid catabolism (Figure 2-3). They may also reflect a decrease in plasma amino acid supply as suggested by the decrease in total plasma amino acid levels (evaluated by the content in total α-NH_2-non-protein nitrogen (αNH_2NPN), i.e., mostly total amino acids), as compared to fed controls [58]. In contrast to fasting, tissue amino acid levels tended to be increased in insulin-neutralized vs. fed, although only glutamine showed a statistically significant response. Comparison of insulin-neutralized vs. fasted chickens highlights the divergent effects of treatments on amino acids (Figure 2-4). Alanine, arginine, asparagine, glutamine, histidine, proline, serine, threonine and tyrosine levels were all significantly higher in insulin-neutralized vs. fasted, with differences ranging from 1.7- to 3.4-fold. Two metabolites related to glucose metabolism, D-glucono-1,5-lactone 6-phosphate and glycerol-3-phosphate, were lower in both fasted and insulin-neutralized treatments vs. fed, with the latter comparison nearing

statistical significance ($p < 0.1$). D-glucono-1,5-lactone 6-phosphate is a product of glucose-6-phosphate dehydrogenase (G6PDH), an enzyme that, in mammals is insulin-sensitive and rate-limiting for pentose phosphate pathway activity and production of cellular NADPH, an important cofactor for lipid metabolism [148]. However, pentose phosphate pathway activity is intrinsically low in chicken and is not stimulated when lipogenesis is high; the production of cellular NADPH is more closely related to malic enzyme activity [149]. Glycerol-3-phosphate is a product of both glucose and pyruvate metabolism and is used in triacylglycerol synthesis. Lower levels with both treatments may reflect glycerol demand for fatty acid reesterification in light of the apparent increase in lipolysis in both treatment groups [58].

Correlated patterns of gene expression and metabolite abundance were extracted using hierarchical clustering to interconnect treatment effects on transcripts and metabolites. Clusters 2 and 3 contained genes and metabolites with lower abundance in fasted vs. fed or insulin neutralized tissue. The two clusters differed with respect to the insulin neutralized group: cluster 3 contained analytes at intermediate levels between fasted and fed, while cluster 2 contained those at levels comparable to or greater than fed. Twelve of the 17 metabolites with statistically suggestive or significant effects of treatment, including all of the amino acids and amino acid derivatives (Additional file 4), were present in cluster 2 along with a set of genes that included the p85α regulatory subunit of PI3 kinase (PIK3R1), as well as ME, malonyl CoA decarboxylase and ELOVL6. Cluster 3 contained several metabolites including both NAD^+ and NADPH and was significantly enriched in GO annotations related to carbohydrate metabolism and in the KEGG pathways TCA cycle, glycolysis/gluconeogenesis, pyruvate metabolism and

steroid biosynthesis ($p < 0.05$; Additional file 4). Clusters 7 and 8 consisted of genes and metabolites with higher levels in fasted than in the other two treatment groups. These clusters were significantly enriched in GO categories PPAR signaling and negative regulation of cellular biosynthesis and also contained citrate and pyruvate ($p < 0.05$; Additional file 4).

Discussion

Despite roles as both a domestic food animal of worldwide economic importance and a widely used model organism with relevance for human obesity and insulin resistance, few studies have examined regulation of gene expression in chicken adipose tissue. To our knowledge, no studies of nutritional regulation of chicken adipose tissue at the genomic level have been reported in the published literature. Likewise, although insulin is the most well-defined hormonal mediator of metabolism in mammalian adipose tissue, its role in chicken remains to be clarified. Therefore the current study addressed two objectives: 1) characterize the transcriptomic and metabolomic response to energy manipulation as a step toward enhanced understanding of adipose biology in chicken; and 2) identify the effects of insulin on chicken adipose tissue by including a group of birds in which insulin action was blocked by immunoneutralization with an anti-insulin antibody. We sought to both identify potential new targets for genetic selection or management strategies to reduce fat accumulation in commercial broilers and to further develop chicken as a model organism for studies of human obesity.

Although intrinsic lipogenic activity is low in chicken adipose tissue, genes involved in fatty acid synthesis and storage were suppressed and those in fatty acid

mobilization and oxidation were up-regulated by fasting. The 40 down-regulated genes with fold changes greater than three were significantly enriched for the GO annotation lipid biosynthetic process (FDR <0.05), including genes that control triglyceride synthesis (DGAT2 and AGPAT9) and fatty acid synthesis (ACACA, ACLY and ME), elongation (ELOVL6), and desaturation (FADS1). AGPAT9 and DGAT2 catalyze the initial and final steps, respectively, of *de novo* triglyceride synthesis. ACLY is the main enzyme for synthesis of cytosolic acetyl-CoA, which is carboxylated to malonyl-CoA by ACACA, the rate-limiting step in fatty acid synthesis. Reducing equivalents for the conversion of malonyl-CoA to palmitate are supplied by malic enzyme (ME). ELOVL6 catalyzes elongation of palmitate to stearate and appears to play a key role in insulin sensitivity [150, 151]. Finally, FADS1 is rate-limiting for polyunsaturated fatty acids (PUFA) biosynthesis and was recently implicated in control of fasting glucose homeostasis in humans [152]. Genes altered by fasting in adipose tissue in this study overlapped with those shown to be differentially expressed in chicken liver after 16 or 48 hours of fasting, including ACLY, ACOX1, BCAT1 and PDK4 [153]. These authors used a different array platform than ours, which precludes precise quantitative comparisons.

Table 2-4 Unique effects of insulin neutralization on differential gene expression

Up-regulated genes			Down-regulated genes		
Gene Symbol	**Gene Title**	**Fold change**	**Gene Symbol**	**Gene Title**	**Fold change**
GCG	Glucagon	2.91	LOC425670	glucagon receptor precursor	2.77
TCP11L2	t-complex 11 (mouse)-like 2	2.08	BAK1	BCL2-antagonist/killer 1	1.83
LOC416916	hypothetical LOC416916	1.98	CCT3	chaperonin containing TCP1, subunit 3 (gamma)	1.62
MAGI1	membrane associated guanylate kinase, WW and PDZ domain containing 1	1.84	SETD7	SET domain containing (lysine methyltransferase) 7	1.62
SEPT10	septin 10	1.79	ST13	suppression of tumorigenicity 13 (colon carcinoma) (Hsp70 interacting protein)	1.62
LSM14A	LSM14A, SCD6 homolog A (S. cerevisiae); similar to LSM14 homolog A (SCD6, S. cerevisiae)	1.71	AHSA1	AHA1, activator of heat shock 90kDa protein ATPase homolog 1 (yeast)	1.62
			TOE1	target of EGR1, member 1 (nuclear)	1.59
			AZIN1	antizyme inhibitor 1	1.56

FDR p-value <0.05

Table 2-5 Fold change of fasting and insulin neutralization on adipose tissue metabolites

Metabolite	Fasting/Fed	Ins-Neu/Fed	Ins-Neu/Fasting
Adenosine	0.57	1.21	2.12*
Alanine	0.66	1.57	2.38***
Arginine	0.75	1.34	1.78**
Asparagine	0.47***	1.00	2.15***
D-glucono-d-lactone-6-phosphate	0.86	0.75*	0.87
Glutamine	0.48***	1.64*	3.43****
glycerol-3-phosphate	0.82	0.72*	0.87
Histidine	0.64	1.38	2.17***
Hypoxanthine	1.31	0.80	0.61**
N-acetyl-glutamate	0.64	1.34	2.09*
N-acetyl-L-serine	0.23**	1.70	7.30***
Ornithine	0.77	1.59	2.05*
Proline	0.63	1.50	2.39***
Serine	0.67	1.61	2.39***
Threonine	0.77	1.38	1.80**
Tyrosine	0.74	1.30	1.76**
a hexose-phosphate	1.05	1.32**	1.26*

* $p < 0.10$, ** $p < 0.05$, *** $p < 0.01$, **** $p < 0.001$

However, among the genes changed in both studies, the fold changes observed in adipose tissue were consistently greater than those in liver, despite the longer duration of fasting in that study. For example, PDK4 expression was up-regulated ~ 18-fold by a five hour fast in adipose tissue, but only ~ 1.5-fold after a 16 hour fast in liver. While differences in sensitivity between the two array platforms must be kept in mind, these data suggest that adipose tissue metabolism in chicken is at least as sensitive to energy status as hepatic metabolism. Our results indicate that both fatty acid synthesis and storage are dynamically regulated by energy status in chicken adipose tissue, despite its modest (~ 15%) contribution to the amount of stored fatty acids.

Both fasted and insulin-neutralized birds exhibited significant increases in plasma glucagon. Parallel elevations in plasma NEFA suggested that this resulted in significant lipolysis of stored triacylglycerol in both treatment groups. During fasting, a considerable percentage of the liberated fatty acids are re-esterified in adipocytes, and only a small fraction traditionally have been thought to be oxidized in the mitochondria of adipocytes through beta oxidation [154]. However, recent studies in mice and in human adipose tissue demonstrate that in some conditions fatty acid oxidation in white adipose tissue is considerable and may be an important determinant of obesity [155-157]. Consistent with this concept, we found significant increases in a number of key enzymes that mediate mobilization of fatty acids and their oxidation, including the rate-limiting enzymes in both mitochondrial and peroxisomal fatty acid oxidation (CPT1A and ACOX1, respectively). We measured tissue levels of beta-hydroxybutyrate, a ketone product of beta oxidation, to confirm that changes in gene expression had functional consequences and found them to be significantly elevated in adipose tissue of fasted vs. fed chickens.

Levels were numerically but not statistically higher in insulin-neutralized adipose tissue (data not shown). Qualitatively, fasting-induced changes in gene expression resemble those induced by the fibrate class of drugs, which activate PPARα and promote fatty acid oxidation in white adipose tissue and are used clinically to treat hyperlipidemia [155, 157-160]. These data suggest that dietary activation of PPARα, for example through supplementation with fatty acids that preferentially bind and activate this member of the PPAR family [161], may be a means to attenuate fat deposition in commercial broilers. Such action may underlie the reduced abdominal fat mass reported in broilers that were fed diets rich in n-3 PUFA[162].

Both fasting and insulin neutralization elicited marked upregulation of PDK4. PDK4 is a nutrient sensing fuel switch that phosphorylates and inactivates pyruvate dehydrogenase, which shifts fuel use from glucose to fatty acids and spares glucose for the brain during periods of fasting [143, 163-165]. PDK4 also enhances glycerol synthesis in white adipose tissue by shunting pyruvate into glyceroneogenesis, at least in the fed state [166]. Hepatic and skeletal muscle expression of PDK4 is increased by fatty acids, acetyl CoA, NADH and the diabetic state and decreased by insulin and pyruvate (rev. in [143, 167]). Little is known about PDK4 in chicken, but a recent study suggests it acts as a glycogen sensor in muscle and thus plays comparable roles to those in mammals [168]. In mouse white adipose tissue, PDK4 expression was shown to be induced by activation of p38MAPK [169], which we found to be significantly up-regulated with fasting and, to a lesser extent, with insulin neutralization [132]. Although PDK4 was up-regulated in both treatment groups, and both groups showed evidence of increased lipolysis [58], only fasted chickens presented a gene expression signature and tissue beta-

hydroxybutyrate levels that were clearly indicative of fatty acid oxidation. Although we did not measure malonyl-CoA levels, we predict that they were reduced with fasting, but not insulin neutralization, based on reduced expression of ACACA. Malonyl-CoA allosterically binds and inhibits CPT1A, minimizing fatty acid transport and subsequent oxidation in mitochondria [170]. With insulin neutralization, increased PDK4 may thus be more aligned with the demand for glycerol needed to reesterify fatty acids liberated by lipolysis [166]. Additional experiments are needed to confirm that manipulation of PDK4 alters fatty acid oxidation in chicken adipose tissue and to delineate its relative contributions to fatty acid oxidation and glyceroneogenesis under varying metabolic states. If manipulation of PDK4 does alter fatty acid oxidation, our results highlight this pathway as a potential target for reducing fatness, which has relevance for both poultry and humans.

Microarray data indicate that the effects of fasting in chicken adipose tissue extend beyond metabolism. GO analysis highlighted pathways such as cell cycle and cytokine-cytokine receptor interaction that are most likely related to changes in the stromal vascular fraction, which contains proliferating preadipocytes and cells of the immune system. In particular, a number of genes that regulate multiple steps in adipogenesis were significantly altered by fasting. Chickens rapidly accumulate abdominal fat after hatch, and until approximately 7 weeks of age this is due more to formation of new adipocytes than to adipocyte hypertrophy [127]. Adipocytes arise from mesenchymal stem cells in a two stage process of lineage commitment to an adipocyte fate, followed by differentiation of fibroblast-like preadipocytes into mature fat-storing cells [171]. Members of both the Wnt (MSC lineage commitment) and TGFβ/BMP (MSC

lineage commitment and preadipocyte competence) signaling pathways were significantly regulated by fasting. Fasting down-regulated expression of CEBPα and PPARγ, two transcription factors that orchestrate the cascade of gene expression changes that lead to terminal adipocyte differentiation [171]. Expression of other adipogenic mediators including fibroblast growth factor 2 (FGF2), fibroblast growth factor receptor 1 (FGFR1) [172], and nuclear receptor corepressor 1 (NCOR1) [173] were also significantly regulated by fasting. Collectively, these changes suggest that adipocyte number in chickens is dynamically tied to energy status, at least in young chicks (such as those used herein) that are rapidly forming new adipocytes. An elegant study by Arner et al. concluded that adipocyte number in humans is a major determinant of adult fat mass and is determined during early childhood [174]. Less is known about this process in humans due to the limitations of sampling adipose tissue, particularly during development and from different abdominal depots. In light of what appears to be sensitive regulation of adipogenesis by nutritional state, chickens may thus be particularly valuable models in which to elucidate mechanisms of adipocyte hyperplasia during development that would inform the study of human obesity.

It is worth noting that, despite the uncertainty about insulin signaling in chicken adipose tissue, fasting altered the expression of several messengers encoding elements of the insulin signaling cascade. Expression of PIK3CB, which encodes the catalytic p110 subunit of PI3K, was up-regulated with fasting, while PIK3R1, which encodes the regulatory p85 subunit, was down-regulated. Such regulation could maintain some insulin signals despite a fall in plasma insulin level. CBLB and CRK, which mediate insulin signals that are associated with lipid rafts [175], were also up-regulated with fasting. In

mammals, this pathway stimulates glucose uptake independently of PI3K activation, which may shed light on the apparent refractoriness of PI3K activity to insulin that was described in chicken skeletal muscle [58]. Therefore, the potential effects of insulin on lipid storage and energy utilization appear to be defended in the fasting state, when insulin levels fall, by enhanced insulin sensitivity at the post-receptor level. Additional studies are needed to confirm this effect and to further explore the potential of PI3K-independent effects of insulin on glucose utilization in chicken adipose tissue.

Insulin is not considered to be a key regulator of glucose metabolism in chicken adipose tissue, although it does induce glucose disposal in chicken liver and muscle [62]. It is therefore not surprising that the majority of genes significantly altered by both insulin neutralization and fasting are not related to glucose metabolism and lipid synthesis. The main exception is DGAT2, which catalyzes the final step in esterification of fatty acids into triglycerides. In fact, DGAT2 showed the most extreme down-regulation (6.1- and 10.5-fold, insulin-neutralized and fasted, respectively) in each treatment group, which is surprising considering that other genes related to lipogenesis were only regulated by fasting. Suppression of DGAT2 expression may be due to feedback by lipolysis, which appeared to be increased in both treatment groups based on plasma NEFA levels. In general, our data indicate that insulin deprivation altered fatty acid and glucose metabolism in a manner comparable to fasting but to a lesser extent, such that most genes involved in these pathways did not exhibit statistically significant changes in expression. For example, cluster analysis (Figure 3-2) revealed that some genes upregulated by fasting were also increased by insulin neutralization (clusters 1, 3 and 4); these three clusters were enriched with genes in the KEGG pathways for fatty

acid metabolism and PPAR signaling, including both ACOX1 and CPT1A, among others. Similarly, among genes that were downregulated by fasting, clustering discriminated a set of genes (Figure 3-2, cluster 2) with a trend to also be decreased (albeit to a lesser extent than in fasting) by insulin deprivation. Interestingly, this cluster was significantly enriched in functions related to carbohydrate metabolism, suggesting that insulin does play some role in chicken adipose glucose metabolism. Comparable trends appeared in the metabolomic data. For example, stearate and palmitate (the only fatty acids measured by our MS platform) were lower (although not significantly) in both fasted and insulin neutralized compared to fed birds (Additional file 3). While the purpose of our study design was to determine the specific effects of insulin on chicken adipose tissue, we cannot exclude the possibility that some of the overlapping changes in gene expression were secondary to systemic factors, such as hyperglucagonemia present in both treatment groups [58]. In vitro experiments using primary adipocytes or adipose explants will be useful to confirm specific effects of insulin on genes identified herein.

Of the 13 changes in expression that were unique to insulin neutralization, the most interesting responses were up-regulation of GCG, which encodes preproglucagon (fold change = 2.91), and down-regulation of the glucagon receptor (LOC425670, fold change = −2.77). The proglucagon system in avians is more complex than in mammals. The avian preproglucagon locus encodes two distinct precursor proteins that yield different peptides through alternative posttranslational processing: the class A transcript (PGA) yields glucagon and glucagon-like peptide-1 (GLP-1), while the class B transcript (PGB) additionally produces glucagon-like peptide-2 (GLP-2) and is more like the mammalian transcript (rev. in [176]). Adipose tissue expresses both transcripts, with

PGA being slightly more abundant, and is the third highest preproglucagon expressing tissue in chicken, behind pancreas and the proventriculus [177]. We used transcript-specific QPCR to determine that only the PGB transcript was up-regulated by insulin neutralization (data not shown). Additional experiments are necessary to delineate which of the encoded peptides are up-regulated in parallel, but the coincident down-regulation of the glucagon receptor suggests a paracrine glucagon axis in chicken adipose tissue, and one that is regulated by insulin. In support of this concept, plasma glucagon (presumably derived largely from pancreas) was elevated comparably in both treatment groups [58], while GCG expression in adipose tissue was only up-regulated by insulin neutralization.

Tissue metabolomic analysis highlighted effects of insulin neutralization that were divergent from fasting and not readily apparent from microarray data. Most of the tissue amino acids that were measured were higher with insulin-neutralization but lower with fasting when each group was compared to *ad libitum* fed controls. This pattern parallels the levels of αNH_2NPN levels in blood [58]. Low levels in fasted adipose tissue were most likely due to oxidation of the carbon skeletons for cellular energy through the tricarboxylic acid cycle (TCA) cycle and/or for glyceroneogenesis, in the absence of dietary glucose. Increased amino acid catabolism was reflected in the differential expression profiles of the fasted vs. fed comparison (Figure 2-3; Additional file 1). In the insulin neutralized group, however, glucose supply from food was maintained and preferentially oxidized for energy. Elevated amino acids in the insulin neutralized group may also reflect reduced utilization due to the lack of insulin's anabolic effects, particularly on the proliferating cell population within adipose tissue. The metabolomics approach used here measured only metabolite pool sizes at the time that tissues were

harvested, rather than the effect of fasting or insulin neutralization on the rates of metabolism through glycolysis and the TCA cycle. The latter would be much more informative with respect to the dynamic impact of treatment, but requires the use of isotopic labeling (e.g., by feeding ^{13}C-labelled glucose) which was not performed in this study. Nonetheless, we were able to demonstrate significant effects on some metabolites that inform the parallel changes in gene expression, particularly in relation to amino acid metabolism. Combined clustering of metabolomic and gene expression together identified a set of genes correlated with many amino acid levels, including PIK3R1, ME and MCD.

Conclusions

In summary, we determined that adipose tissue metabolism in the chicken is regulated by energy status and, to a lesser extent by insulin. Although adipose tissue is not a primary site of lipogenesis in chicken, the rate-limiting genes for fatty acid synthesis were suppressed by fasting. Likewise, fasting appeared to increase aspects of insulin sensitivity based on expression profiles, despite the view that chicken adipose tissue is relatively insensitive to insulin. Consistent with this paradigm, insulin neutralization significantly altered the expression of only a few genes related to glucose and lipid metabolism. Nonetheless, a considerable number of genes were altered by insulin neutralization, many of which thus far have unclear roles in adipose biology. Expression profiles suggest that even short term fasting alters fat storage in broilers by enhancing the oxidation of fatty acids. The initiating events that trigger upregulation of the corresponding genes are unclear, but there is considerable evidence for activation of

PPARa, LXRa, and potentially other transcription factors that are activated by fatty acid ligands. Further studies are warranted to identify these triggers because of their potential impact on fat storage. Our data also suggest that broiler chicks may be an informative model organism in which to investigate dietary effects on adipose development in light of what appears to be a relationship between energy intake and adipogenesis. The results of this study thus have dual benefit for both the poultry industry and for studies of obesity in humans.

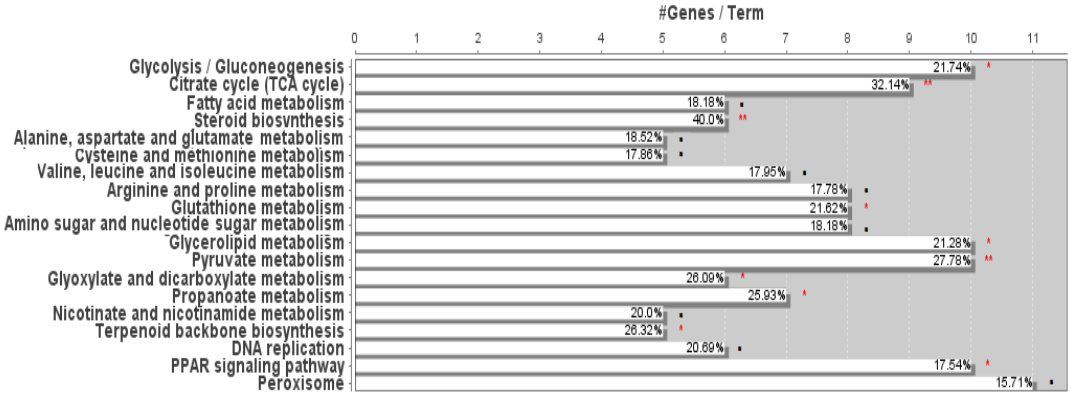


Figure 2-3 KEGG pathway analysis of genes differentially expressed in fasting vs. fed. Genes differentially expressed in fasting vs. fed were matched to KEGG pathway membership using ClueGO. The percentage and #genes/term indicates the percentage and number of the genes in the pathway that are contained in the set of genes altered by fasting. • $p < 0.1$ * $p < 0.05$ **, $p < 0.01$, based on Benjamini-corrected p-value

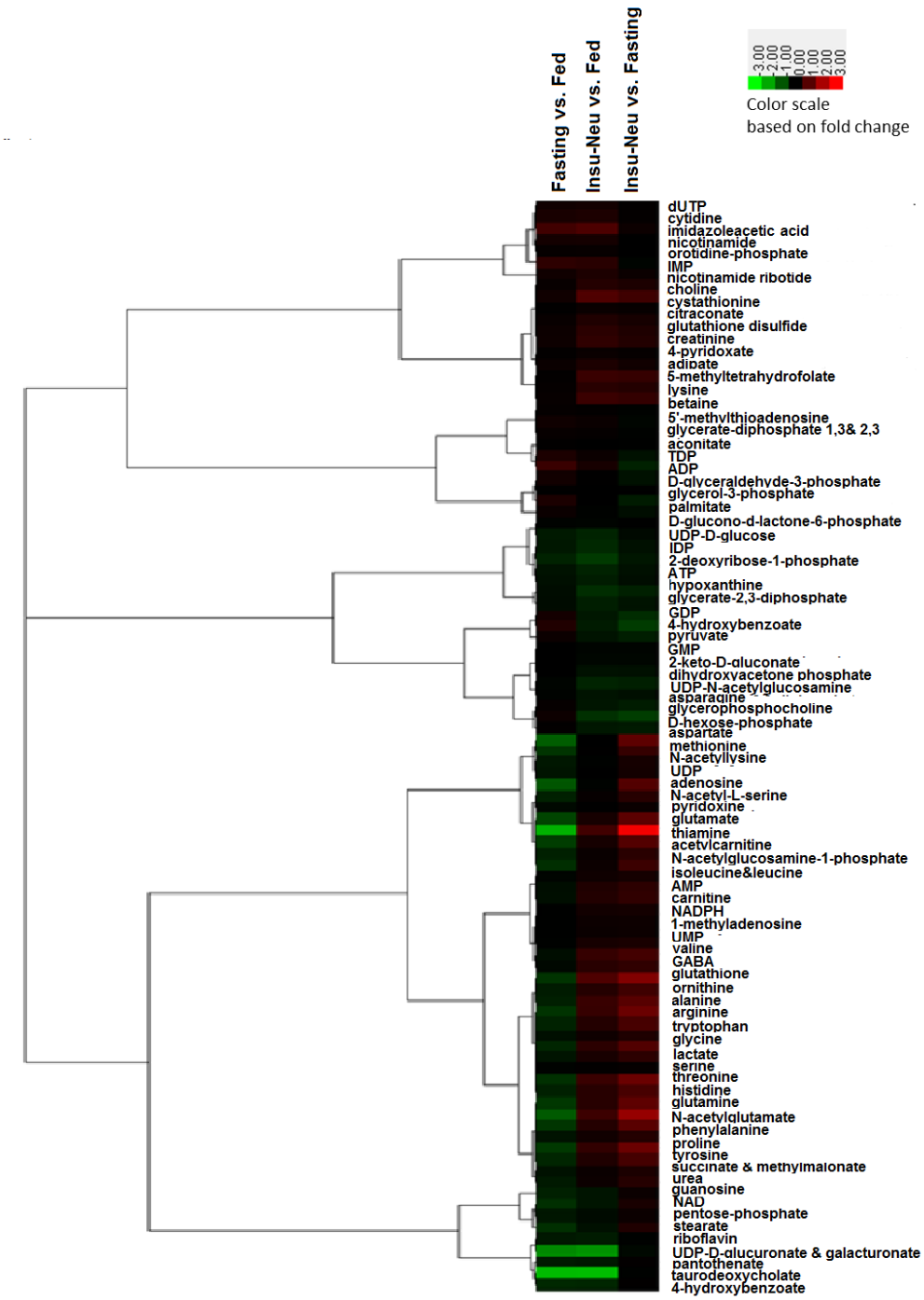


Figure 2-4 Heat map of metabolites. The median value of each metabolite in each treatment group was used to calculate fold-change of fasted vs. fed, insulin-neutralized (insneut) vs. fed, and insulin-neutralized vs. fasted, and then values were subjected to hierarchical clustering.

CHAPTER III

GENETIC LEANNESS IN DOMESTIC CHICKENS IS ASSOCIATED WITH INCREASED FATTY ACID OXIDATION IN WHITE ADIPOSE TISSUE

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Bo Ji, Jesse L. Middleton, Ben Ernest, Arnold M. Saxton, Susan J. Lamont, Shawn R. Campagna and Brynn H. Voy

Introduction

Domestic chicken has been intensively studied because of its role as an efficient source of lean meat. However, commercial broilers resulting from genetic selection for rapid growth demonstrate detrimental traits, such as excess deposition of abdominal adipose tissue, metabolic disorders, and reduced reproduction [178]. Of interest, such traits are also associated with human obesity [179]. Therefore, fast-growing broilers (meat-type) represent "obese" chickens compared to slow-growing egg layers (e.g, Leghorn) or wild-type strain chickens (e.g., Fayoumi) (rev. in [120]). Fayoumi chickens, originating from Egypt, represent a hardier stain of chickens that is more resistant to diseases [180]. Fayoumi has fast maturity and lays small eggs, which makes it a layer mostly raised in farms. Leghorn chickens are the original breed of commercial U.S layers. Both lines were maintained highly inbred by Iowa State University poultry geneticists with an inbreeding coefficient higher than 0.95. Both Fayoumi and Leghorn demonstrated lean phenotype compared to broilers, and these three lines of chickens are genetically distant from each other [181]. Specifically, chicken F2 populations generated from commercial broilers male line and two genetically distinct inbred lines (Leghorn

and Fayoumi) provide unique models through which to integrate phenotypes with genotypes. Genome-wide linkage analyses on F2 populations have identified QTLs associated with abdominal fat percentage [182], skeletal integrity, and metabolic traits in the chickens [111-114]. However, the genomic characterization of adipose tissue from the parental lines has not been studied yet.

Additionally, study of egg-laying chickens provides insights to understand lipid mobilization, transfer and utilization for yolk precursor synthesis, which is important in deposition of yolk and embryo development [183]. Chickens also possess unique features, including natural hyperglycemia (up to 200 mg/dL in the fasting state) and resistance to exogenous insulin [121, 122], which mimic early stage of type 2 diabetes in humans. Most of the genes located the quantitative trait loci (QTLs) regions for the fatness trait were found to be obesity and diabetes related genes in humans [115-117]. Unlike rodents, *de novo* lipogenesis in adipose tissue is relatively low in chickens and humans [123-125], which implicate the similarity of adipose metabolism between chickens and humans. Likewise, adipose cellular development is characterized by hyperplasia early in life through posthatching to first several weeks of age [78], and it paralleled hypertrophy through adulthood, and predominant hypertrophy at an older age. This correlates with the observations in humans that adult-onset obesity is associated with increased adipocyte size, whereas early-onset obesity has both adipocyte hypertrophy and hyperplasia [80].

Previously, we identified pathways related to adipose metabolism, signaling and adipogenesis by characterizing the response of chicken adipose tissue to short-term feed restriction, which is a management practice in broiler industry to control fatness [184]. In

the present study, we assessed the physiological, transcriptomic and metabolomics variations on adipose tissue of three genetically distinct lines of chickens: Fayoumi (M 5.1), Leghorn (Ghs-13) and commercial broilers. We aimed to 1) understand the adipose physiology of genetically distinct lines of chickens; 2) identify genes and pathways associated with the leanness of wild chickens, which are potential targets for control of fatness in broilers through either genetic selection or improved management practices. Plasma metabolites and endocrine parameters were investigated and abdominal adipose tissue transcriptomics and metabolomics were assayed by microarrays and LC-MS. The association of gene expression, metabolic and physiological responses was performed to provide a comprehensive understanding of the variation in fatness between broiler, Fayoumi and Leghorn.

Methods and Materials

Animals

Male birds from broiler, Leghorn (Ghs-13) and Fayoumi (M5.1) lines maintained at the Iowa State University were sacrificed at week 7 by our collaborator in Dr. Lamont's lab who provided tissue samples. All hatched chicks (inbred Leghorn, inbred Fayoumi, and broilers) were wing-banded for individual pedigree identification. Birds were grown under standard management conditions from hatch to 7 weeks of age and had *ad libitum* access to water and feed.

Determination of physiological parameters

Body weight and fat weight were measured at week 7. Abdominal adipose tissue and serum samples from six birds of each line were collected and rapidly snap-frozen in liquid nitrogen, then stored at -80^{o}C until analysis. Serum glucose levels were measured

by the glucose oxidase method using glucose colorimetric kit (Cayman, MI, USA). Free or non-esterified fatty acid (NEFA) levels and triglyceride (TAG) were determined using an enzymatic colorimetric kit (Wako Chemicals, Neuss, Germany). Glucagon and VLDL levels in the serum were determined by chicken glucagon ELISA kit and chicken VLDL ELISA kit (Novatein Biosciences, MA, USA). ß-hydroxybutyrate (b-HB) was determined by enzymatic colorimetric kit (Biovision, CA, USA). Lipid peroxidation was determined by measuring thiobarbituric acid reactive substrates (TBARS) (Cayman, MI, USA).

Histological analysis of adipocyte size

A portion of the abdominal fat pad was fixed in 4% paraformaldehyde then washed in PBS. The samples were embedded in paraffin, cut into 5µm sections, and stained with hematoxylin and eosin. Adipocyte size was measured using a previously established method [185]. Briefly, the tissue sections were viewed at 10X magnification, and images were obtained. The illumination of the images was adjusted using CellProfiler cell image analysis software (www.cellprofiler.org). After the illumination was adjusted, the images were converted to a binary image in ImageJ (NIH; rsbweb.nih.gov/ij/) using the following commands: Gray Mode and Threshold. The binary images were further adjusted using Despeckle, Watershed, Erode, and Paintbrush. The cross-sectional area of the adipocytes was measured using the "Analyze particles" command. Approximately 200 adipocytes per chicken were measured.

Gene expression

Total RNA was isolated from chicken adipose samples using the RNeasy Lipid kit and incorporating an on-column DNase treated with the RNase-free DNase Set according to the manufacturer's protocol. RNA quality and concentration was measured using the Experion System (BioRad.com); only RNA passing recommended standards of quality

was used for further studies. Transcriptome profiling in chicken adipose tissue was performed by Genome Science Resource at Vanderbuilt University (Nashville, US) using the Affymetrix GenChip chicken genome array on the Affymetrix platform (San Diego, CA). For real time PCR validation, cDNA was synthesized using the iScript cDNA Synthesis kit. Commercially designed and validated primer sets (QuantiTect) primers were used in conjunction with the QuantiTect SYBR Green PCR kit on a CFX96 real-time PCR detection system (Bio-Rad.com). RNA isolation and quantitative PCR (QPCR) reagents were obtained from Qiagen (qiagen.com). All samples were analyzed in triplicate and normalized to ß-tubulin. Relative differences in gene expression were determined using the ΔΔCT method ($2^{-\Delta\Delta CT}$ as the fold change) and statistical differences were tested by analysis of variance (ANOVA) [134].

Liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS)

Abdominal adipose tissue samples from six birds of each line (the same six birds used for expression profiling) were extracted by placing tissue in a mortar containing liquid nitrogen and then powdering with a pestle. Portions (8 - 40 mg) of the powered tissue were weighed into 1.5 mL centrifuge tubes. Chilled methanol (0.3 mL at -80 ^{o}C) and internal standard (5 µL of 1.7 mM benzoic acid in negative mode or 4.25 mM tris (hydroxymethyl) aminomethane in positive mode) were added to each tube. Each tube was mixed thoroughly by vortexing for two minutes, and the metabolites were extracted from the tissue for 30 min at 4 ^{o}C. The tubes were then centrifuged (5 min, 4 ^{o}C, 16.1 rcf) and supernatant was removed. The extraction procedure was repeated for three times. Supernatant was split into two autosampler vials. One of these samples was immediately placed on the LC-MS/MS for analysis, while the other was stored at -80 ^{o}C for analysis in

the opposite polarity ion mode on the following day. The samples were placed in an autosampler tray chilled to 4 ^{o}C, and 10 µL of each was injected onto an LC column for analysis as described previously.

Statistical analysis

Statistical analysis of the microarray data was performed using R 2.13.0 and routines contained in Bioconductor (bioconductor.org) as described in previous study [184]. Statistical analysis of metabolomic data was performed using an analysis tool that we developed specifically for metabolomic data analyses [140]. The script (metabR), written in the language R, uses linear mixed-effect modeling to normalize metabolomics data for fat mass and internal standard . The script averages any replicate measurements (statistical sampling) made on experimental units and performs ANOVA to test for statistical differences between experimental groups. Body weight, fat weight, adipocity, and plasma levels of glucose, triglyceride, NEFA and TBARs were compared among groups using Tukey test in SAS 9.0. Distribution of adipocyte sizes was analyzed using propTable and plotted in R 2.13.0.

Results

As expected, measures of fatness differ significantly between the three lines. Broilers are significantly fatter than Fayoumi and Leghorn in terms of body weight, fat weight, and adiposity (Table 3-1). The relationships between the physiological parameters were demonstrated using Pearson correlation coefficient (Table 3-2). Adipocyte size increased with fatness and differed significantly between each pairwise comparison, with the largest fat cells found in broilers and the smallest in Leghorn, the leanest of the three lines (Figure 3-1A). The number of very large adipocytes (>10000 um^2) was about 8% in the tissue of broilers, but less than 1% in Fayoumi and Leghorn

(Figure 3-1B). Plasma levels of non-esterified fatty acids (an index of lipolysis) and β-hydroxybutyrate (a systemic measure of fatty acid oxidation) were significantly higher in lean chickens, i.e., Leghorn and Fayoumi, than broilers ($p < 0.05$). Circulating VLDL levels were more than three times greater in broilers vs. Fayoumi, consistent with elevated VLDL in genetically selected fat lines of chickens [23, 24]. However, plasma level of VLDL in Leghorn was similar to broilers. Thiobarbituric acid reactive substances (TBARs), byproducts of lipid peroxidation, were higher in Fayoumi and Leghorn than broilers, but not statistically different. Plasma glucose and glucagon levels did not differ significantly between the three lines of chickens (data not shown).

Physiological differences among the lean lines compared to broilers are paralleled by significant differences in adipose gene expression profiles. Expression levels of a total of 2361 genes were differentially expressed in Leghorn and/or Fayoumi compared to broilers based on an FDR adjusted p-value < 0.05 (Additional file 1; Figure 3-2A). Ninety-three percent (2212 of 2361) of these genes showed a fold-change > |1.5| (Figure 3-2B). The number of genes that were differentially expressed in Fayoumi vs. broiler and Leghorn vs. broiler are essentially equivalent (1179 and 1354 genes, respectively).

Eleven genes of interest were selected based on fold-change or biological functions of interest based on our prior fasting study (Figure 3-2). Differential expression of PDK4, DUSP5, CCL20, TLR4, AGTR1 and NAMPT in Leghorn versus broilers was identified. Genes that were differentially expressed in Leghorn compared to broilers were also differentially expressed in Fayoumi except for AGTR1 based on real-time RT-PCR. All of the genes confirmed by QPCR as increased in lean vs. fatty lines were previously shown to be up-regulated in fasted vs. fed adipose tissue.

Hierarchical cluster analysis of the 2361 genes that were differentially expressed in at least one pairwise comparison was used to visualize the similarities and differences between groups. As shown in Figure 3-4A, birds within each of the three genetic lines clustered together. The dendrogram also shows that expression profiles in broiler adipose tissue were distant from those of Fayoumi and Leghorn, which were more similar to each other. Eight clusters representing different patterns were identified in terms of gene expression (Figure 3-4B). Four clusters (1, 3, 4 and 7) were of interest because their expression patterns qualitatively correlated with fatness (Figure 3-4). The genes in each cluster were submitted to Gene Ontology analysis, which provides the biological interpretation of the clusters and identifies significantly over-represented GO terms and pathways (Table 3-3).

Cluster 1 contains genes expressed at higher levels in Fayoumi and Leghorn vs. broiler. This set of genes was significantly enriched in endocytosis, regulation of Ras protein signal transduction, fatty acid metabolic process and regulation of catabolic process. Cluster 7 contains genes of the inverse pattern, with lower expression in Fayoumi and Leghorn compared to broiler. These genes are enriched in steroid biosynthesis, oxidative phosphorylation and negative regulation of low-density lipoprotein particle receptor biosynthetic process.

Cluster 3 consists of genes with the highest expression in broiler and the lowest expression in Leghorn, corresponding to the two extremes in terms of degree of fatness. These sets of genes were significantly enriched in functions related to lipid biosynthetic process, cholesterol metabolic process and lipid metabolic process (Table 3-2). Contrary to cluster 3, cluster 4 contained genes with highest expression in Leghorn and lowest in

broilers. This set of genes represents a number of functional annotations, including calcium ion transport and cellular calcium ion homeostasis.

GO enrichment was used to functionally annotate the set of 1233 genes that were differentially expressed between broilers and Leghorns, the leaner of the two lean lines. The biological process GO terms were used to characterize the genes differentially expressed in Leghorn versus broilers based on FDR<0.05 and absolute fold-change ≥1.5 (Figure 3-5A). Leghorn differed from broilers in a diverse set of processes related to lipid and fatty acid metabolism and synthesis. This set of processes includes fatty acid and lipid biosynthetic processes, sterol and cholesterol biosynthetic and metabolic processes, fatty acid oxidation, lipid modification, fat cell differentiation, carboxylic acid catabolic process. Within these categories, the expression of genes involved in fatty acid oxidation (e.g, acetyl-CoA carboxylase beta (ACACB), carnitine palmitoyltransferase 1A (CPT1A), protein kinase, AMP-activated, alpha 1 catalytic subunit (PRKAA1)) were up-regulated in Leghorn compared to broilers, while expression of genes that control fatty acid, sterol, cholesterol and triacylglycerol synthesis were down-regulated (e.g., Diacylglycerol O-acyltransferase homolog 2 (DGAT2), 3-hydroxy-3-methylglutaryl-Coenzyme A reductase (HMGCR), stearoyl-CoA desaturase (SCD), farnesyl diphosphate synthase (FDPS), and acetoacetyl-CoA synthetase (AACS), fatty acid desaturase 1 and 2 (FADS1 and FADS2)). However, we also found that some genes in fatty acid oxidation (ACAD11, ACOX2) were down-regulated in Leghorn. Leghorn also showed up-regulated expression of genes related to the regulation of adipogenesis (e.g., lipin 1 (LPIN1), laminin alpha 4 (LAMA4), WNT inhibitory factor 1 (WIF1), transcription factor 7-like 2 (TCF7L2), tribbles homolog 2 (TRIB2), frizzled-related protein (FRZB),

aldehyde dehydrogenase 6 family (ALDH6A1)). Leghorn also had a lower expression of genes involved in antigen processing and presentation (e.g., major histocompatibility complex (MHC) Rfp-Y class I alpha (YFVI), MHC class I antigen (YFV), MHC class II antigen B-F minor heavy chain; MHC class II beta chain (BLB1), similar to MHC Rfp-Y class I alpha MHC I-related (MR1)). The diversity of MHC was identified to be responsible for the genetic resistance to pathogens in chickens [186]. This is also consistent with the previous findings that Leghorn line was the most susceptible to pathogens [180].

Genes in many of the same pathways were also differentially expressed in Fayoumi vs. broilers. Genes that differed between these two strains, based on FDR<0.05 and absolute fold-change ≥1.5, were significantly enriched in fatty acid and lipid catabolic process, lipid modification, fatty acid oxidation, carboxylic acid catabolic process (Figure 3-5B). Among these categories, the genes that were up-regulated are involved in fatty acid oxidation (ACACB, ACAD11, ACOX2, EHHADH, PHYH, PRKAA1).

Metabolic intermediates that were significantly altered in Leghorn and Fayoumi compared to broilers were identified. A total of 92 metabolites were detected based on spectral peak area (in ion counts) (Table 3-5). A total of 47 metabolites were different between treatment groups based on adjusted p<0.05. Metabolic intermediates of glycolysis/gluconeogenesis pathways, including phosphoenolpyruvate and 3-phosphoglycerate, were significantly higher only in Leghorn vs. broilers (p<0.05), although the levels of glucose-6-phosphate, glycerol-3-phosphate and pyruvate were similar in Leghorn chickens vs. broilers. The accumulation of phosphoenolpyruvate and

3-phosphoglycerate may reflect the decrease in glycolysis or increase in gluconeogenesis in Leghorn chickens.

Some metabolic intermediates (carnitine, acetylcarnitine, pentose phosphate, glycerophosphocholine, and UDP-N-acetylglucosamine (GlcNAC)) involved in lipid and carbohydrate metabolism were significantly higher in both Fayoumi and Leghorn, as compared to broilers. In contrast to Leghorn, tissue levels of amino acids were higher in Fayoumi vs. broilers, with statistically significant increase of threonine, proline, valine and ornithine. It is worth noting that NADPH, the reducing agent in lipid biosynthesis, was significantly decreased in Fayoumi vs. broilers ($p<0.05$). Only 11 metabolites were significantly different in Fayoumi and Leghorn. Five metabolites (threonine, pantothenate, pyruvate, aspartate, and ox._Glutahion) were lower in Leghorn vs. Fayoumi, but the others (asparagine, 1-Methylhistidine, NADPH, Ethanolamine_or_Urea, Hypoxanthine_(NEG), 3-Phosphoglycerate) were higher in Leghorn.

Discussion

Although attention has been drawn to domestic chickens because of their role as an important food animal, few studies have examined the transcriptomic and metabolomics differences of adipose tissue between different strains of chickens, specifically, egg-laying, meat-type chickens, and broilers. The primary objective of our present study was to define the transcriptomic and metabolomic characterization of distinct genetic lines of chickens that present different phenotypic traits to further understand adipose biology in domestic chickens. Secondly, we aimed to identify the association of genomic responses in adipose tissue with physiological and metabolic responses in chicken to solidify the status of chicken as a model organism for studies of

human obesity. We expected that the differences in gene expression and metabolites of three lines of chickens might explain the distinct phenotypic differences in the fatness of broilers and wild chickens.

The lean phenotypes of Fayoumi and Leghorn were regulated by genes involved in various pathways and biological processes related to lipid and fatty acid biosynthesis and metabolism (Figure 3-6). Lipid accumulation in adipose tissue of chicken was considered partially through uptake of lipoproteins assembled in liver [41]. The candidate receptors responsible for lipid accumulation in adipose tissue include LDL receptor (LDLR), VLDL receptor (VLDLR) and LDL receptor-related protein (LRP) [187]. Our data demonstrated that the expression of LDLR and VLDLR were down-regulated in Leghorn vs. broiler. Apolipoprotein A1 (APOA1), a major protein component of high density lipoprotein (HDL) [36], was found to be significantly up-regulated in Leghorn. And LRP and VLDLR were down- regulated in Fayoumi. These results indicated there might be lower uptake of VLDL-triglycerides in adipose tissue of lean chickens compared to broilers. VLDL and vitellogenin (VTG) are major yolk precursors. Egg-laying hens exhibit high plasma levels of VLDL and VTG at the onset of egg laying [188]. Therefore, the down-regulated VLDL receptor in adipose tissue of egg-type chickens was probably to accumulate VLDL in plasma.

Genes differentially expressed in adipose tissue of Leghorn in this study overlapped with those altered by 5 hour fasting in adipose tissue of broilers, including genes enriched in lipids and cholesterol biosynthesis and fatty acid oxidation pathways [20]. The genes enriched in lipid biosynthetic process were down-regulated, but in fatty acid oxidation were up-regulated in white adipose tissue of Leghorn compared to broilers.

Acetoacetyl-CoA synthetase (AACS) was down-regulated (2.1- fold) in Leghorn vs. broiler. AACS catalyzes the formation of acetoacetyl CoA, which is the precursor of HMG-CoA, initializing cholesterol synthesis. Additionally, a number of genes (ACAT2, FDPS, IDL1, SQLE, LSS, SC4MOL, NSDHL, HSD17B7, DHCR7, DHCR24) involved in the whole mevalonate pathway were significantly down-regulated in Leghorn vs. broiler. Diacylglycerol kinase, theta (DGKQ) was down regulated (4.3-fold) in Leghorn vs. broiler. DGKQ is a member of diacylglycerol kinases family that metabolize 1,2,diacylglycerol (DAG) to produce phosphatidic acid (PA). Together with the down regulation of DGAT2 and elevated tissue level of glycerolphosphocholine, our results indicate that the energy consumed may contribute to the synthesis of phosphatidylcholine by increasing glycerolphosphocholine instead of TAG or PA in adipose tissue of Leghorn.

Interestingly, we found PDK4, one of the most markedly up-regulated genes by 5 hour fasting in adipose tissue of chicken, was greatly up-regulated (5.5- fold) in Leghorn vs. broiler. PDK4 phosphorylates and inactivates pyruvate dehydrogenase, functioning as the fuel switch that shifts fuel use from glucose to fatty acids during fasting. PDK4 may also contribute to glyceroneogenesis or gluconeogenesis by shunting pyruvate for glycerol synthesis or enhancing substrate for gluconeogenesis [189, 190]. Increased tissue levels of phosphenolpyruvate and phosphoglycerate, intermediates of glyceroneogenesis and gluconeogenesis pathways, confirmed this concept. Thus, upregulation of PDK4 in both lean chickens and fasted chickens indicate that PDK4 could be a potential target to manipulate for reducing fatness in chickens. Expression of other genes related to fatty acid oxidation, such as lipin-1 and CPT1A, were significantly up-regulated in Leghorn vs. broilers based on fold-change. Lipin-1 was recently shown to amplify PPARα and Pgc-1α

activity, further enhancing fatty acid oxidation in liver [191]. Additionally, elevation of tissue levels of carnitine and acetylcarnitine in lean chickens further confirmed increased fatty acid oxidation. Carnitine and acetylcarnitine, the activated form of carnitine, play an important role in fatty acid oxidation by transporting long-chain acyl groups from fatty acids into the mitochondrial matrix. This process is accomplished by the carnitine palmitoyltransferase (CPT) system. CPT1A is rate-limiting enzyme initiating the mitochondrial oxidation of long-chain fatty acids. Consistent with upregulation of genes and metabolites in fatty acid oxidation, we also found elevations in plasma NEFA and B-hydroxybutyrate, which suggested elevated lipolysis and fatty acid oxidation in lean chickens.

In addition to fatty acid metabolism, the cluster of genes enriched in calcium ion transport and cellular calcium ion homeostasis represent the unique characterization of Leghorn as a layer line. Differential expression of this set of genes may result from the selection on higher egg and eggshell weight in layers, which require high amounts of calcium for the formation of eggshells [192].

In contrast to Leghorn, genes (HIBCH, ALDH2, ALDH3A2, ALDH6A1, EHHADH, MCD, MUT, SUCLG2) enriched in functions related to propionate metabolism were up-regulated in Fayoumi vs. broilers. Enoyl-CoA, hydratase/3-hydroxyacyl CoA dehydrogenase (EHHADH), which is an L-bifunctional enzyme involved in classical peroxisomal fatty acid β-oxidation pathway, was recently recognized as indispensable enzyme for the production of medium-chain dicarboxylic acids [193]. Malonyl-CoA decarboxylase (MCD), the enzyme responsible for decarboxylation of malonyl-CoA to acetyl-CoA, was significantly up regulated (3.78-fold)

in Fayoumi. Increased MCD activity resulted in an elevated rate of fatty acid oxidation in liver and heart by rapidly decreasing malonyl –CoA level [194-196]. Methylmalonyl CoA mutase (MUT) and succinate-CoA ligase (SUCLG2) are responsible for conversion of propanoyl-CoA (a product of beta-oxidation of odd-chain fatty acids or BCAA) to succinyl-CoA for energy [197]. Similarly, Hydroxyisobutyryl-CoA Hydrolase (HIBCH) catalyzes the pathway for the metabolism of branched-chain amino acid (BCAA) and propionate, indicating the increased proteolysis to provide energy. A recent study demonstrated that SUCLG2 and HIBCH were up regulated in the lipodystrophic mice model [198]. Aldehyde dehydrogenases (ALDHs), a superfamily of NAD(P)(+)-dependent enzymes with similar primary structures, catalyze the oxidation of a wide spectrum of endogenous and exogenous aliphatic and aromatic aldehydes to fatty acids [199]. Three genes (ALDH2, ALDH3A2, ALDH6A1) encoding enzymes of ALDHs family were up-regulated in Fayoumi. Aldehyde dehydrogenase enzymes produce NADPH, which is consistent with higher levels of NADPH in Fayoumi vs. broilers. The substrates of ALDHs are the products of lipid peroxidation, therefore elevated ALDHs may be due to higher lipid peroxidation, which was confirmed by higher serum TBAR level in Fayoumi chickens. However, some of these genes (EHHADH, MCD, HIBCH, and ALDH2) were down-regulated in Leghorn, as compared to broilers. The distinct regulation of the same genes in propionate metabolism pathway in Fayoumi and Leghorn reflected their preference for energy sources. Leghorn mainly utilized energy from oxidation of long chain fatty acids instead of oxidation of carbohydrate, whereas Fayoumi consumed energy from oxidation of medium chain fatty acids and/ or metabolism of amino acids from elevated proteolysis.

Gene expression profiling of adipose tissue indicated that the features that distinguished the three lines of chickens from each other are not limited to metabolism. In particular, GO analysis demonstrated that genes were enriched in fat cell differentiation pathway in Leghorn. Mesenchymal stem cells (MSC) give rise to adipocytes by lineage commitment, preadipocytes proliferation, and differentiation. CEBPa, the transcription factor that triggers the adipocyte differentiation, was significantly down-regulated in Leghorn vs. broiler. However, adipose differentiation-related protein (ADFP) was significantly up-regulated in Leghorn. ADFP is specific marker for lipid accumulation, whereas, it was also found to be induced by long chain fatty acid, PPARa and fasting in liver [200, 201]. The expression of adipogenic mediators, including fibroblast growth factor 2 (FGF2) and fibroblast growth factor receptor 3 (FGFR3), was significantly down-regulated in Leghorn compared with broilers. Additionally, the substantial up-regulation of Wnt inhibitory factor 1 (WIF1, 44.8-fold) in Leghorn vs. broiler indicates the inhibition of Wnt (MSC lineage commitment) signaling in Leghorn [202]. In contrast, these genes ((WIF1), adiponectin (ADIPOQ), and chibby (CBY1)) were down-regulated in Fayoumi [203]. In agreement with data of adipocyte sizes, Leghorn showed dramatic changes of genes in the development of adipocytes, while the changes in Fayoumi were minimal. It worth noting that transcription factor 7-like 2 (TCF7L2) was significantly differentially expressed in both Leghorn and Fayoumi vs. broilers. TCF7L2 was found to be associated with type 2 diabetes in adults and impaired glucose metabolism in children [204]. This implicates the potential application of chicken as a model to study human obesity and diabetes.

Leghorn and Fayoumi demonstrated different expression of genes related to insulin signaling cascades. Expression of inositol hexakisphosphate kinase (IP6K2) was up-regulated in Leghorn vs. broilers. Expression of PIK3CB and PIK3C2A, which encode isoforms of class I and class II of PI3Ks respectively [205], were up-regulated in Fayoumi vs. broilers. Expression of PRKAA1 and PRKAB2, which encode catalytic and regulatory subunits of the 5 prime AMP-activated protein kinase (AMPK), were up-regulated (21- and 2- fold, respectively) in Fayoumi vs. broilers, whereas only PRKAA1 was up-regulated in Leghorn. AMPK plays a major role in ACC and MCD regulation by phosphorylating and inactivating ACC activity, but activating MCD activity, which favors fatty acid oxidation [206]. Thus, the marked increase of PRKAA1 may be responsible for the transcriptional alteration of genes in fatty acid oxidation in lean chickens. A recent study found activation of AMPK inhibits PPARa and PPARy transcriptional activity in hepatoma cells [207], which may result in insignificant changes of PPARa and PPARy in lean chickens.

In summary, we determined that adipose tissue metabolism in lean chickens is distinct from that in broilers. The leanness of Leghorn (layer) and Fayoumi (meat type) were regulated by various pathways related to lipid and fatty acid biosynthesis and adipogenesis. Expression profiles indicated that fatty acid metabolism was altered from storage toward oxidation in lean chickens. Our data also suggest the differences in adipose metabolism between layers and meat type chickens. This study is beneficial to both obesity study and poultry science.

Table 3-1. Physiological parameters of three lines of chickens.

	Broiler	Fayoumi	Leghorn
body weight (g)	2208.2 ±214.1[a]	524.1 ±35.5 [b]	415.19 ±18.2 [b]
fat weight (g)	20.0 ±5.6[a]	3.3 ±1.8[b]	1.5 ±0.8[b]
Fat/BW	0.009 ±0.0002[a]	0.006 ±0.0002[b]	0.004 ±0.00018[c]
non-esterified fatty acid (mM)	0.21 ±0.03[c]	0.41 ±0.07[a]	0.29 ±0.04[b]
triglyceride (mg/dL)	57.99 ±14.8[b]	98.96 ±32.3[a]	56.55 ±25.6[b]
ß-hydroxybutyrate (mM)	0.34 ±0.05[c]	0.57 ±0.07[a]	0.44 ±0.05[b]
TBARS (uM)	2.2 ±0.7[a]	3.1 ±1[a]	2.6 ±0.7[a]
very low density lipoprotein (ug/ml)	0.085 ±0.027[a]	0.024 ±0.0025[b]	0.070 ±0.019[a]
glucagon (pg/ml)	76.7 ±11.6[a]	82.6 ±20.2[a]	84.1 ±5.5[a]

Table 3-2 Correlation of physiological parameters

Variable	by Variable	Correlation	p-value
adiposity	fat weight	0.58	0.02278
ß-hydroxybutyrate	Triglyceride	0.73	0.00187
ß-hydroxybutyrate	body weight	-0.69	0.00406
ß-hydroxybutyrate	fat weight	-0.63	0.01263
fat weight	body weight	0.94	1.1E-07
NEFA	adiposity	-0.73	0.00188
NEFA	fat weight	-0.57	0.02712
TBARS	ß-hydroxybutyrate	0.53	0.04326
VLDL	TBARS	-0.63	0.01126
VLDL	fat weight	0.62	0.01324
VLDL	body weight	0.56	0.02868

Table 3-3. Gene ontology (GO) and KEGG annotation for representative clusters of differentially expressed genes.

Cluster	Annotation	GO term (Biological process, level of 6 or 7) or KEGG pathway name	FDR p-value	Genes
1 (180 genes)	KEGG	Ribosome biogenesis in eukaryotes	9.7 E-3	[HEATR1, REXO1, SBDS, TBL3]
		Melanogenesis	1.2 E-2	[CREB3L2, FZD6, MITF, TCF7L2]
		Endocytosis	3.8 E-2	[BF2, GIT2, RAB22A, VPS4B]
	GO	ncRNA metabolic process	3.9 E-2	[INTS2, INTS6, SBDS, TBL3]
		Regulation of Ras protein signal transduction	4.2 E-2	[ARFGEF1, FGD3, FGD4, GIT2, GPR65]
		Fatty acid metabolic	4.4 E-2	[ACACB, HPGD, LPIN1, PRKAA1]
		Regulation of small GTPase mediated signal transduction	4.6 E-2	[ARFGEF1, FGD3, FGD4, GIT2, GPR65]
		Regulation of catabolic process	5.0 E-2	[ADRA1B, GIT2, GPR65, PRKAA1, UVRAG]
2 (260 genes)	GO	Regulation of sequence-specific DNA binding transcription factor activity	2.0 E-2	[FZD4, HCK, ID2, PRKCH, VEGFA]
		Peptidyl-amino acid modification	2.1 E-2	[ADH5, CD3E, CSRP2BP, HCK, PDK3, PRMT7, VEGFA, WEE1]
		Brain development	3.8 E-2	[FZD4, GPR56, ID2, NPY, SLC4A7, ULK1]
		Peptidyl-tyrosine phosphorylation	4.3 E-2	[CD3E, HCK, VEGFA, WEE1]
		Peptidyl-tyrosine modification	4.3 E-2	[CD3E, HCK, VEGFA, WEE1]
		Regulation of neurogenesis	4.5 E-2	[DLL1, ID2, PRKCH, ULK1, VEGFA]
		Ribonucleotide metabolic process	4.6 E-2	[ATP1B1, GART, TMED2, UMPS]
	KEGG	Phagosome	1.9 E-2	[ATP6V0A1, BLB2, COMP, MARCO]
3 (439 genes	KEGG	Terpenoid backbone Biosynthesis	2.6 E-4	[ACAT2, FDPS, HMGCR, IDI1, PDSS1]

Table 3-3 Continued

Cluster	Annotation	GO term (Biological process, level of 6 or 7) or KEGG pathway name	FDR p-value	Genes
		Steroid biosynthesis	7.9 E-4	[DHCR7, LSS, NSDHL, SC4MOL]
		Metabolic pathways	3.2 E-3	[ACAT2, ACLY, ACOX2, ACSS3, AK2, ALDH2, ALDH4A1, ALG11, ATP6V0E1, DEGS1, DGKQ, DHCR7, EXTL3, FDFT1, FDPS, HMBS, HMGCR, HPRT1, IDI1, LSS, MMAB, NADSYN1, NDUFA11, NDUFB2, NSDHL, OXSM, PAPSS1, PLA2G6, PLCD1, PRDX6, QDPR, SC4MOL, SQLE, WBSCR17]
	GO	Lipid biosynthetic process	5.5 E-3	[DEGS1, DHCR7, FDFT1, FDPS, HMGCR, IDI1, LSS, NSDHL, OXSM, PDSS1, PLA2G6, SC4MOL, SCD, ST8SIA6]
		Cholesterol metabolic process	8.8 E-3	[CEBPA, DHCR7, FDPS, HMGCR, LSS, NSDHL]
		Lipid metabolic process	2.9 E-2	[AACS, ACAD11, ACOX2, CEBPA, DEGS1, DHCR7, FDFT1, FDPS, HMGCR, IDI1, LSS, NSDHL, OXSM, PDSS1, PLA2G6, PLCD1, PRDX6, SC4MOL, SCD, ST8SIA6]
		Alcohol metabolic process	4.3 E-2	[CEBPA, DHCR7, FDPS, HMGCR, HPRT1, LSS, NSDHL, SNCAIP]
4 (296 genes)	GO	Regulation of transmembrane transport	3.5 E-2	[BCL2, CACNG4, GNA11, TRPV2]
		Calcium ion transport	3.6 E-2	[BCL2, LOC395914, SLC24A2, TRPV2]
		Cellular calcium ion homeostasis	4.3 E-2	[AGTR1, BCL2, LOC395914, SLC24A2]

Table 3-3 Continued

Cluster	Annotation	GO term (Biological process, level of 6 or 7) or KEGG pathway name	FDR p-value	Genes
		Regulation of membrane potential	4.4 E-2	[ADAM22, BCL2, GNA11, UGT8]
		Positive regulation of cell development	4.6 E-2	[BCL2, CDH4, DCT, TRPV2]
5 (300 genes)	KEGG	Biosynthesis of unsaturated fatty acid	1.5 E-3	[ELOVL5, FADS1, FADS2, PTPLB]
		Endocytosis	2.9 E-2	[ACAP2, CHMP1B, EGFR, KIT, LDLRAP1, RCJMB04_3g13, SMAD7, STAM]
6 (151 genes)	GO	Fat cell differentiation	2.1 E-3	[ARID5B, EIF2AK3, LAMB3, SOD2, TCF7L2]
		Regulation of neurological system process	1.0 E-2	[ATAD1, EIF2AK3, LAMA2, TCF7L2]
		Regulation of transmission of nerve impulse	1.4 E-2	[ATAD1, EIF2AK3, LAMA2, TCF7L2]
		Multicellular organismal signaling	3.2 E-2	[ATAD1, CAMK4, EIF2AK3, LAMA2, TCF7L2]
		Transmission of nerve impulse	3.6 E-2	[ATAD1, CAMK4, EIF2AK3, LAMA2, TCF7L2]
		Oxidation-reduction process	4.3 E-2	[NDUFB4, NDUFB8, SOD2, TALDO1]
		Cellular amino acid metabolic process	4.8 E-2	[BCKDHB, BHMT, MUT, SOD2]
7 (473 genes)	KEGG	Steroid biosynthesis	4.2 E-2	[DHCR24, HSD17B7, LIPA]
		Oxidative phosphorylation	5.0 E-2	[ATP5G3, ATP5O, ATP6V1C2, COX6C, COX7A2L, NDUFA10, NDUFA2, NDUFC2]

Table 3-3 Continued

Cluster	Annotation	GO term (Biological process, level of 6 or 7) or KEGG pathway name	FDR p-value	Genes
	GO	Negative regulation of low-density lipoprotein particle receptor biosynthetic process	2.8 E-2	[ADIPOQ, ITGAV]
		Myeloid cell differentiation	3.0 E-2	[ADD1, ADIPOQ, CAMK4, HMGB2, JAG1, LMO2, NDFIP1, PRDX3, TESC]
		Negative regulation of macrophage derived foam cell differentiation	3.3 E-2	[ADIPOQ, ITGAV]
		Regulation of myeloid cell differentiation	3.7 E-2	[ADIPOQ, HMGB2, JAG1, LMO2, NDFIP1, TESC]
8 (262 genes)	KEGG	Lysine degradation	5.0 E-2	[ASH1L, PLOD2, SUV420H1]
		Rgulation of neuron projection development	4.2 E-2	[CCDC88A, GOLGA2, LYN, PSEN1, PTEN]
		Negative regulation of transferase activity	4.5 E-2	[LYN, PRDX3, PSEN1, PTEN]
		Peptidyl-tyrosine phosphorylation	4.7 E-2	[IL31RA, JAK1, LYN, PDGFRA, PSEN1]
		Peptidyl-tyrosine modification	4.7 E-2	[IL31RA, JAK1, LYN, PDGFRA, PSEN1]
		Negative regulation of kinase activity	4.9 E-2	[LYN, PRDX3, PSEN1, PTEN]
		Regulation of neuron differentiation	4.9 E-2	[BMP6, CCDC88A, CDK5RAP2, GOLGA2, LYN, PSEN1, PTEN]

Table 3-4 Correlation of Adipocyte size with 53 common genes identified by three pairwise contrasts.

Gene	Correlation	Signif Prob
PQLC3	-0.95	7.31E-08
SQLE	0.94	1.34E-07
DCT	-0.94	2.19E-07
SYT15	-0.91	3.27E-06
TMEM164	0.89	8.00E-06
LOC100859544	-0.89	1.03E-05
FAS	-0.88	1.24E-05
RDM1	-0.88	1.39E-05
CEP170	0.88	1.48E-05
USPL1	-0.88	1.75E-05
WIF1	-0.86	4.75E-05
IFI27L2	-0.86	4.86E-05
LOC420414	-0.84	7.54E-05
GNL3	-0.84	1.03E-04
CXCR4	0.82	1.75E-04
RASD1	0.81	2.33E-04
LACE1	0.78	6.49E-04
MTMR2	0.71	2.98E-03
NHP2L1	0.70	3.78E-03
MPZL1	0.65	8.60E-03
PON2	0.61	1.58E-02
HEY1	-0.59	1.97E-02
NMRAL1	0.59	2.00E-02
VLDLR	0.58	2.41E-02
DCAF6	-0.58	2.41E-02
ALDH6A1	-0.57	2.54E-02
ERGIC1	-0.52	4.86E-02

Table 3-5 Fold change of adipose tissue metabolites in pairwise comparisons of three strains (broiler, Leghorn, and Fayoumi)

Metabolites	Fayoumi-Broiler	Leghorn-Broiler	Leghorn-Fayoumi
ox._Glutahione_or_DG	5.28***	0.87	0.16**
Threonine_(POS)	4.11***	1.46***	0.36***
Pantothenate	1.65***	0.63***	0.38***
Oxidized_Glutathione_(GSSG)	2.49**	1.20	0.48
Pyruvate	1.43***	0.74	0.52***
1-Methyladenosine	5.76***	3.21***	0.55
Pentose_Phosphate	3.73***	2.09***	0.56
Ornithine	2.65***	1.54	0.58
IMP	0.89	0.53***	0.60
Nicotinamide_Ribotide	1.99**	1.34**	0.67
Aspartate	1.73**	1.23	0.71**
Reduced_Glutathione_(GSH)	1.88**	1.38***	0.73
Ceramide	1.93	1.42	0.74
Lysine-(67)	2.15***	1.62***	0.75
Proline	1.90**	1.45**	0.76
Carnitine	3.44**	2.69***	0.78
Lysine-(84)	1.77**	1.41***	0.80
Valine	1.62**	1.33***	0.82
Acetylcarnitine_DL	3.57***	2.94***	0.82
Deoxyguanosine	2.03***	1.71**	0.84
Guanosine	3.17***	3.03***	0.95
5prime-Methylthioadenosine	2.16***	2.13**	0.98
Glycerophosphocholine	2.48***	2.53***	1.02
Taurine	1.48***	1.59**	1.08
Guanine	2.35***	2.62***	1.11
Creatinine	1.77***	2.03**	1.14
Riboflavin	1.40	1.65**	1.17
N-Acetylglucosamine-1-Phosphate	1.73***	2.10**	1.22
Serine	1.32***	1.64**	1.25
Xanthine_(POS)	1.59**	2.04***	1.29
Lactate	0.78**	1.00	1.29
Malate	1.30	1.81***	1.39
Cytidine	1.44	2.01***	1.39
Nicotinamide	1.07	1.54**	1.43
Asparagine	0.99	1.55***	1.56***
Adenosine	2.19**	3.47***	1.59

Table 3-5 Continued

Metabolites	Fayoumi-Broiler	Leghorn-Broiler	Leghorn-Fayoumi
Cytosine	2.76**	4.39***	1.59
Citrulline	2.06***	3.29***	1.59
Glycerol-3-Phosphate	0.64	1.10	1.71
deoxyribose	1.97**	3.61***	1.83
Ethanolamine_or_Urea	1.91**	4.07***	2.13**
Imidazoleacetic_Acid	2.02	4.33***	2.15
1-Methylhistidine	1.13	2.48***	2.19***
Hypoxanthine_(NEG)	1.33	3.13**	2.36**
3-Phosphoglycerate	0.96	3.46***	3.61**
NADPH	0.31***	1.24	4.02**
Phosphoenolpyruvate	1.39	7.42***	5.33

** $p < 0.05$, *** $p < 0.01$, **** $p < 0.001$

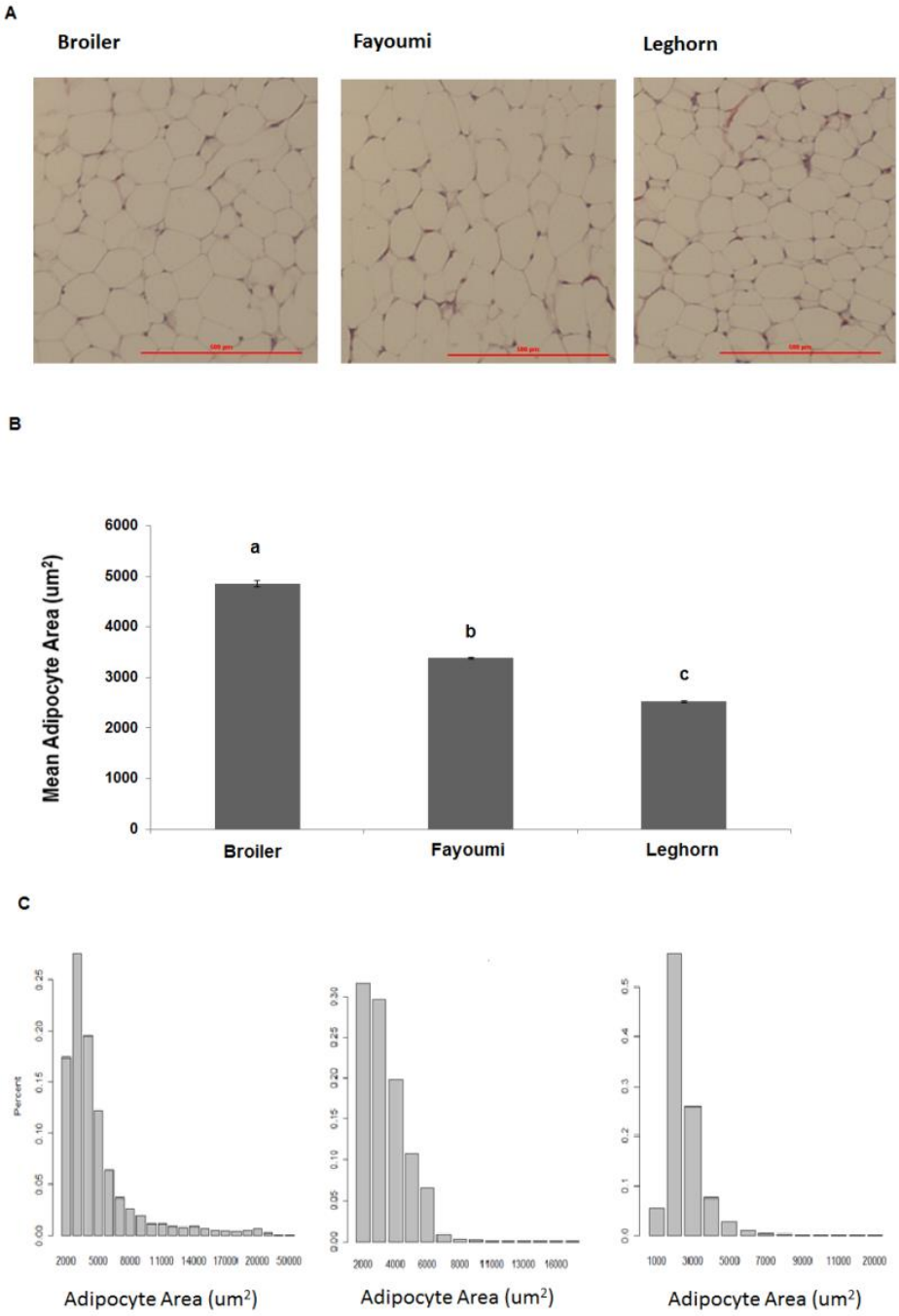


Figure 3-1. A. Histology of abdominal adipose tissue from broiler, Fayoumi and Leghorn. Scale bar: 500 µm. B. Adipocyte area was measured using ImageJ software. Mean adipocyte area in broiler, Fayoumi and Leghorn (n = 7/group). C. the distribution of adipocyte area. When letters above bars differ, means significantly differ by $p<0.05$.

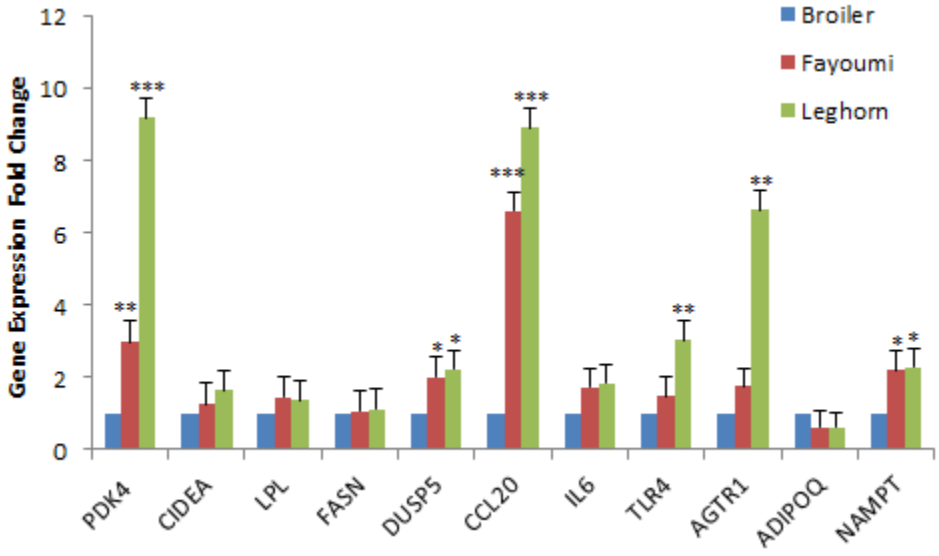


Figure 3-2. QPCR results for selected genes in chickens. Results are expressed as fold change determined using the ΔΔCt method. Blue: broiler, red: Fayoumi, green: Leghorn. *p<0.05, **p<0.01. ***p<0.001

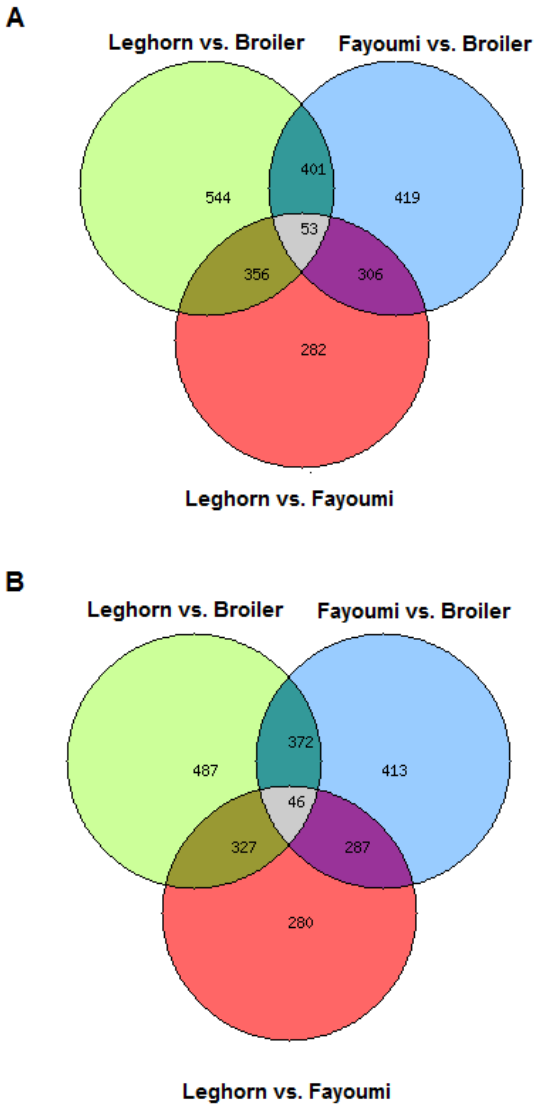


Figure 3-3. Venn diagram of overlapping and unique effects of Leghorn and Fayoumi vs. broiler (a) A total of 2361 genes were differentially expressed (FDR adjusted pvalue <0.05) between one or more pairwise treatment comparisons; (b) A total of 2212 genes with absolute fold change ⩾1.5 among the differentially expressed genes.

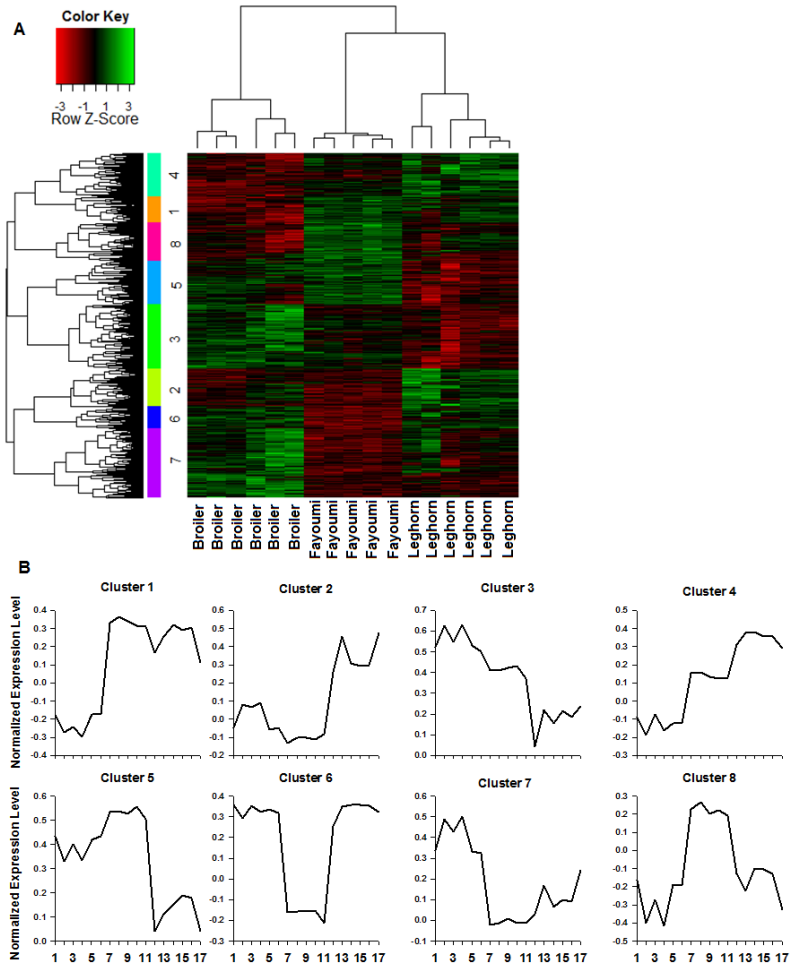
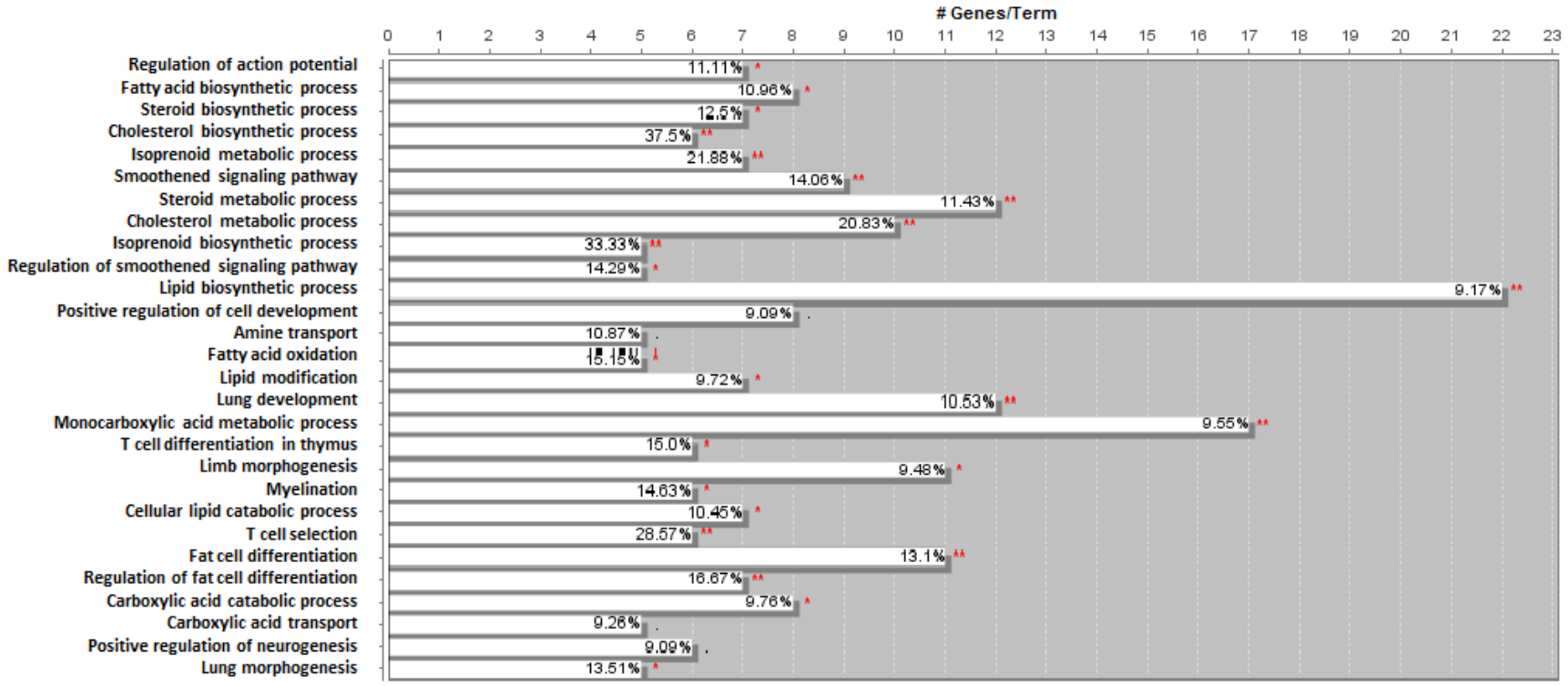
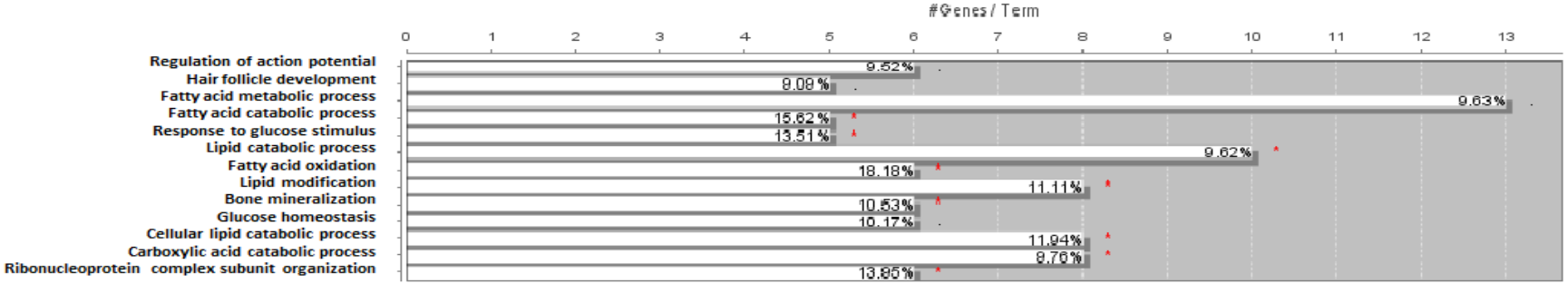


Figure 3-4. Cluster analysis of differentially expressed genes. The 2361 genes differentially expressed in one or both inbred chickens vs. broilers were subjected to hierarchical clustering to visualize similarities and differences between breeds. (a). Hierarchical cluster analysis of the 2361 genes (FDR adjusted pvalue <0.05) that were differentially expressed between Leghorn vs. broiler and/or Fayoumi vs. broilers. (b) Expression profiles of eight clusters extracted by hierarchical cluster. Sample ID number on the X-axis corresponds to treatment group: sample 1-6, broiler; 7-11, Fayoumi; 12-17, Leghorn. Y-axis represents relative gene expression value.

A


Genes/Term
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23
Regulation of action potential
Fatty acid biosynthetic process
Steroid biosynthetic process
Cholesterol biosynthetic process
Isoprenoid metabolic process
Smoothened signaling pathway
Steroid metabolic process
Cholesterol metabolic process
Isoprenoid biosynthetic process
Regulation of smoothened signaling pathway
Lipid biosynthetic process
Positive regulation of cell development
Amine transport
Fatty acid oxidation
Lipid modification
Lung development
Monocarboxylic acid metabolic process
T cell differentiation in thymus
Limb morphogenesis
Myelination
Cellular lipid catabolic process
T cell selection
Fat cell differentiation
Regulation of fat cell differentiation
Carboxylic acid catabolic process
Carboxylic acid transport
Positive regulation of neurogenesis
Lung morphogenesis
11.11% *
10.96% *
12.5% *
37.5% **
21.88% **
14.06% **
11.43% **
20.83% **
33.33% **
14.29% *
9.17% **
9.09% .
10.87% .
15.15% *
9.72% *
10.53% **
9.55% **
15.0% *
9.48% *
14.63% *
10.45% *
28.57% **
13.1% **
16.67% **
9.76% *
9.26% .
9.09% .
13.51% *

B



C

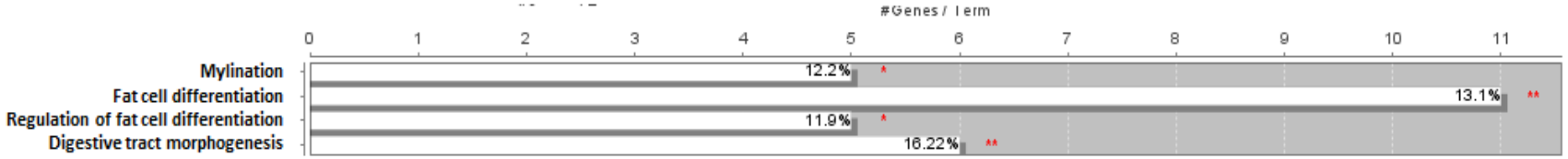


Figure 3-5 KEGG pathways analysis of genes differentially expressed genes. Genes were matched to KEGG pathway membership using ClueGO. (A) Leghorn vs. broiler. (B) Fayoumi vs. broiler (C) Leghorn vs. Fayoumi. The percentage and #genes/term indicates the percentage and number of the genes in the pathway that are contained in the set of genes differentially expressed in different breeds of chicken. • $p < 0.1$ * $p < 0.05$ **, $p < 0.01$, based on Benjamini-corrected p-value

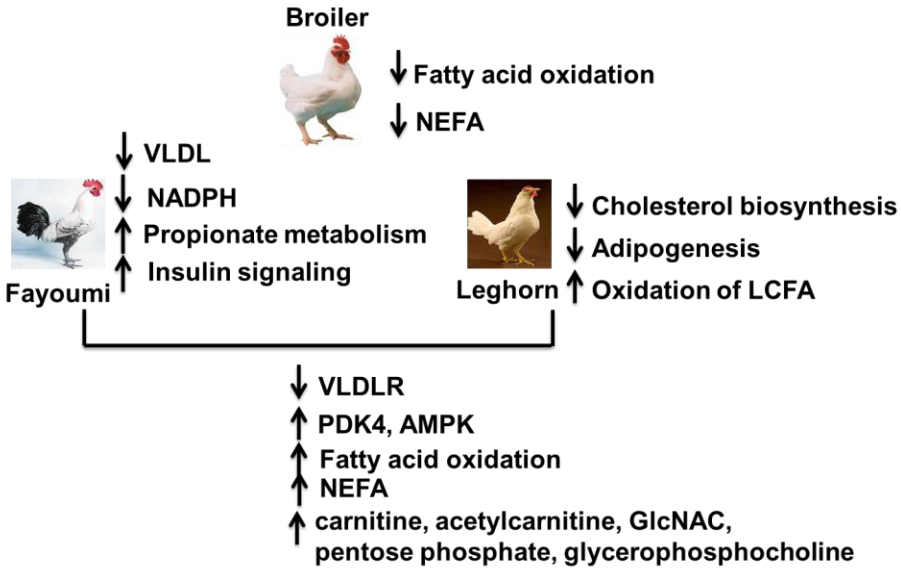


Figure 3-6 Shared traits of naturally lean chickens.

CHAPTER IV

GENE EXPRESSION SIMILARITIES BETWEEN FASTED AND LEAN PHYSIOLOGIES IN DOMESTIC CHICKENS

This manuscript will be submitted to *Poultry Science* with the following author list:
Bo Ji, Ben Ernest, Arnold M. Saxton, Susan J. Lamont, and Brynn H. Voy

Introduction

Obesity in humans may be attributable in part to decreased oxidation of fatty acids in skeletal muscle, the primary contributor to whole body fatty acid oxidation. Fatty acid oxidation is impaired systemically, and specifically in skeletal muscle, in obese vs. normal weight patients [208-210]. Obesity is associated with a variety of metabolic changes, however, it is difficult to determine which is the cause and which is the consequence of the obese state. Nonetheless, some studies suggest that impaired fatty acid oxidation is a primary defect that contributes to the development of obesity. Normal weight subjects with a family history of obesity were shown to have lower rates of fatty acid oxidation than individuals with no family history [211]. Further, normal weight children with an obese parent also exhibit reduced fatty acid oxidation in muscle [212], suggesting a genetic component to fatty acid oxidation that is inked to leanness.

Little is known about the role of fatty acid oxidation in the control of leanness in chickens. In our previous studies (Chapters 2 and 3), we found gene expression profiles indicative of increased fatty acid oxidation in adipose tissue of fasted broilers vs. fed and in genetically lean Leghorn and Fayoumi lines compared to broilers. We therefore reasoned that fasting and genetic leanness may share common features in terms of

adipose tissue metabolism, including increased fatty acid oxidation. To explore this possibility, we conducted a combined analysis of gene expression profiles from the fasting and insulin-neutralization study and from the study of broilers, Leghorn and Fayoumi. The two studies were performed in different countries and with differences in husbandry, age of animals and other unknown confounds. We also compared the effects of fasting and leanness on adipose metabolism signalling pathways using Western blot analyses and on the fatty acid composition of adipose tissue using GC-MS. Part of the Western blot results described in this chapter were previously published in [68].

Methods

Quantification of fatty acids with gas chromatography/mass spectrometer (GC/MS)

Fatty acids samples were prepared for GC-MS analysis following the steps of lipid extraction, triglyceride hydrolyzation, esterification and methylation of fatty acids according to the methods described [208-210]. Nonadecanoic acid was used as an internal control. Approximately 10-50 mg adipose tissue was homogenized using 2:1 chloroform:methanol. Lipids were extracted in the organic phase by centration. Lipids were hydrolyzed using 0.3 M KOH in methanol and incubated at 80℃ for 60 minutes. Boron trifluoride (BF3, 12-15%) in methanol was used as an acid catalyst for esterification and transesterification. In esterification, a methyl group from methanol is added to the carboxyl group of free fatty acids at 90℃ for 90 minutes. Fatty acids were reconstituted in hexane for GC-MS analysis. The fatty acid methyl ester was analyzed using a gas chromatography system, Agilent 6890 GC G2579A system (Agilent)

equipped with an OMEGAWAX 250 capillary column (Supelco) and a flame ionization detector. An Agilent 5973 mass selective detector was used to identify target peaks.

Western blotting

Western blotting was used to determine the effects of fasting and insulin neutralization on insulin and stress signaling by investigating the protein levels of phosphorylated and total AKT, ERK, AMPK, S6K, mTOR, p38 (Cell Signaling). Protein lysates was prepared by tissue extraction buffer (Invitrogen) supplemented with protease and phosphatase inhibitor (Roche). Proteins were detected using ECL Plus chemiluminescent substrate (gelifesciences.com) and intensity of signals following hybridization was measured using a chemiluminescent detector (Versadoc; bio-rad.com).

Statistical analysis

Statistical analysis of the microarray data was performed using R 2.13. Data normalization and processing was performed as described previously [184]. Statistical significance of gene expression differences were analyzed by two-way ANOVA (treatment (T) and country (C) factors) and empirical bayes using the limma package. Differential expression was defined based on false discovery rate (FDR) adjusted p-value <0.05. Venn diagrams of differentially expressed genes were plotted to visualize the number of differentially expressed genes for each factor and interaction of factors. Hierarchical clustering of significant genes was performed using the hclust function and a hierarchical clustering heatmap was created using heatmap.2 in the gplots package.

The model (Equation 1) was based on defining treatment level 1 as fasted chickens (France) and lean Leghorn (US), and treatment level 2 was broilers from both countries. Country factor represent the variations associated with geographical and husbandry effects.

$Y_{ij}=\mu+T_i+C_j+T*C_{ij}$ (1)

The difference between birds for each fatty acid was evaluated by analysis of variance (ANOVA) and post hoc Tukey test for multiple comparisons in SAS (SAS Institute, Cary, NC). The correlation coefficients of phenotypic traits and gene expression were based on Pearson correlation.

Results

Mega-analysis of Affymetrix data from different studies

Two-way ANOVA model identified that 1587 genes were differentially expressed in terms of treatment, and 1919 genes in terms of country factor, and 390 genes affected by the interaction of treatment and country based on an FDR adjusted p-value < 0.05. The genes of interest are 1049 genes (954 genes of them with fold change> 1.5) that were only altered by treatment, but not by country and/ or interaction of treatment and country (Figure 1A).

Hierarchical cluster analysis was employed based on 1049 genes that were only significantly altered by treatment factor. As shown in Figure 1B, broilers fed *ad libitum* were clustered with broilers, whereas fasted birds were clustered together with lean Leghorn chickens. Five clusters representing different patterns were identified in terms of gene expression denoted by the color bar (Figure 1B). The genes in cluster 1 (493 genes) were of interest because they showed very consistent low expression in lean and fasted chickens, but high expression in broilers. These genes were submitted to DAVID Gene Ontology analysis, which provides the biological interpretation of the clusters and identifies significantly over-represented GO terms and pathways (Table 1).

Effects of fasting and insulin neutralization on phosphorylation of Akt, MAPKs ERK1/2, mTOR, S6K, AMPK, and P38MAPK in chicken adipose tissue

The effects of fasting and insulin neutralization on phosphorylation of Akt, AMPK, MAPKs ERK1/2, mTOR, S6K and P38 insulin signaling pathways were evaluated in chicken adipose tissue (Figure 4-2). Our results suggested that phosphorylation of Akt, ERK1/2, S6K were decreased by fasting and insulin-neutralization in chickens. However, there was no significant effect of fasting and insulin-neutralization on phosphorylated level of mTOR and AMPK. Interestingly, fasting significantly increased MAPK P38 phosphorylation in chicken adipose tissue.

Phosphorylation of Akt, MAPKs ERK1/2, S6K, AMPK, and P38MAPK in adipose tissue of lean and broilers

Our data suggested that the phosphorylated levels of Akt, MAPKs ERK1/2, S6K and P38MAPK were similar between Leghorn, Fayoumi and broilers. Nevertheless, phosphorylated level of AMPK was higher in Fayoumi vs. broilers (Figure 4-3).

Effect of fasting and insulin neutralization on compositions of fatty acids

Compositions of most fatty acids were not altered by fasting and insulin neutralization, except for cis-vaccenic (18:1(n-7)) and linoleic acid (18:2 (n-6)), which were statistically significant lower in fasted vs. fed chickens ($p<0.05$). However, absolute concentrations of fatty acids are consistently lower in fasted chickens than in fed chickens (data not shown). There was no significant difference of fatty acid compositions in insulin neutralized chickens compared to *ad libitum* fed chickens.

Compositions of fatty acids in Leghorn, Fayoumi and broilers

Oleic acid (18:1 (n-9)), a monounsaturated fatty acid, is the most abundant fatty acid in chicken adipose tissue, accounting for about 40% of total fatty acids. The

abundance of oleic acid was significantly lower in Leghorn than broilers ($p<0.05$). Compositions of some other fatty acids, including myristic acid (14:0), palmitic acid (16:0), stearic acid (18:0), and linoleic acid (18:2 (n-6)), were significantly higher in Leghorn vs. broilers and Fayoumi ($p<0.05$). Consequently, the ratios of saturated fatty acids to unsaturated fatty acids were significantly higher in Leghorn vs. broilers and Fayoumi ($p<0.05$). Interestingly, docosahexaenoic acid (DHA, 22:6 (n-3)) was significantly higher ($p<0.05$) in Leghorn vs. Fayoumi, as was the ratio of EPA and DHA to AA, which is the ratio of n-3 to n-6. In contrast, Fayoumi had similar compositions of fatty acids as broilers, except that Fayoumi had the highest abundance of oleic acid, and the highest ratio of n-3 to n-6 among the three lines of chickens.

Correlation of physiological responses and gene expression

The correlations of physiological responses to fasting and insulin neutralization and gene expression were investigated. Variables with correlation coefficients higher than 0.7 ($p<0.001$) were considered as strongly correlated. Plasma triodothyronine (T3) was highly correlated with expressions of a large number of genes (901 genes). Plasma glucose was highly correlated with 140 genes. Plasma glucagon and NEFA were highly correlated with 143 genes and 525 genes, respectively. Some of the genes were both highly correlated with plasma levels of hormones and metabolites (Figure 4-4). Interestingly, eight unique genes that are only highly correlated with glucagon include GCG, ST13, and TCP11L2, which were also identified as uniquely differentially expressed in insulin neutralized chickens.

Discussion

We sought to identify similarities in gene expression signatures between leanness and feed restriction by integrating the array data generated from previous studies.

However, the true effect of the treatment was confounded by geographical and husbandry factors associated with these distinct studies. To account for these confounding experimental factors, we first developed a linear model to identify the effect of the confounding factor and its interactive effect with treatment, then excluded the genes that were affected by the confounding factor and its interaction with treatment. The genes that were only affected by treatment factor were submitted for hierarchical clustering and Gene Ontology analysis. Two-way ANOVA model identified the similarities between fasting broiler and naturally lean chickens. Cluster 1 contained genes with lower abundance in Leghorn and fasted chickens vs. broilers were significantly enriched in fatty acid biosynthetic and metabololic process, steroid and carboxylic acid biosynthetic process (Benjamini adj. pvalue<0.05). Cluster 2 and 3 consisted of genes with higher expression in lean and fasted chickens compared to broilers. These clusters were enriched in genes involved in immune responses and in fatty acid oxidation, including PDK3, PDK4, ACACB, and CPT1A. This method increased power and accuracy to detect differentially expressed genes in fasted broilers and lean chickens compared to fed broilers, regardless of breeds, geographical and husbandry factors. Furthermore, it identified the similarities of fasted broilers and naturally lean Leghorn lying in the categories characterized by decreased fatty acid biosynthesis and increased fatty acid oxidation.

Chickens are considered to be refractory to insulin. Chicken muscle showed insulin resistance, but only in the early steps of insulin signaling [58, 67]. Moreover, genetically fat and lean line chickens showed apparent difference in insulin signaling in response to feeding, prolonged fasting and refeeding. Fat line chickens exhibited higher

insulin sensitivity in liver than lean line chickens in terms of early steps (IRß, IRS-1, Shc and PI3K) of insulin signaling cascade, which would account for increased liver lipogenesis in fat line chicken [64]. Nevertheless, little difference was observed in leg muscle of fat line chickens regarding the early steps of insulin signaling in response to nutritional status, which may be due to consistent refractory to insulin in muscles [64]. However, little is known about chicken adipose tissue. In this study, we assessed phosphorylated levels of insulin signalling cascades in chickens from both studies to characterize the effects of fasting and insulin on signalling pathways, and insulin signalling cascades in different breeds of chickens.

In terms of the effects of fasting on insulin signaling pathways, the genes up or down stream of AKT, ERK and mTOR signaling pathways were significantly differentially expressed in fasted chickens, such as DUSP5, MYC, MAP3K5. Consistently, decreased phosphorylated levels of AKT, ERK, mTOR and p70S6K were observed in fasted chickens. AMPK, which is considered as energy sensor, is activated in response to nutrient deficiency or stress to support cellular metabolism and maintain the ATP cellular level. However, the activation of AMPK by 5-hour fasting was not significant (Fig. 3). For the stress signaling, TLR4 and TNFR6, which are up-stream of JNK signaling pathways were up-regulated. It is consistent with the activation of p38 and indicated the promotion of inflammation in adipose tissue through the released fatty acids by fasting. As described in Chapter 3, we found that Expression of PRKAA1 and PRKAB2, which encode catalytic and regulatory subunits of AMPK, were up-regulated (21- and 2- fold, respectively) in Fayoumi vs. broiler adipose tissue. Correspondingly,

phosphorylated level of AMPK was higher in Fayoumi than broilers, whereas the difference was not observed in Leghorn chickens.

AMPK plays a major role in ACC and MCD regulation by phosphorylating and inactivating ACC activity, but activating MCD activity, which favors fatty acid oxidation [206]. Together with increased expression of genes involved in fatty acid oxidation, activation AMPK further implicated favored fatty acid oxidation in lean chickens. Additionally, PDK4 expression was shown to be induced by activation of p38MAPK in mouse white adipose tissue [169], which was also observed in adipose tissue of fasted and lean chickens.

Like humans, but unlike rodents, oleic acid is the most abundant fatty acid in adipose tissue of chickens[211]. Linoleic acid (18:2) is an essential fatty acid not synthesized in the body of animals, which is derived from diet. Linoleic acid was higher in Leghorn, which only indicated the differences in their feed uptake. Leghorn had less unsaturated fatty acids and more saturated fatty acids than broilers, which is different from previous studies on mice. Obese mice had significant increases in saturated fatty acids, and a significantly decrease in unsaturated fatty acids. The opposite result in lean chicken is propably due to the egg-laying feature of Leghorn, which have their specific features of lipid homeostasis and fatty acids transfer to maintain follicular cells and form the yolk. Another reason for increased saturated fatty acids may be due to their different expression of fatty acid elongase. Fatty acid elongase 6 (ELOVL6) is the rate-limiting elongase related to *de novo* lipogenesis. Pigs carrying the allele associated with a decrease in ELOVL6 gene expression had increased C16:0 and C16:1 (n-7) fatty acid content and a decrease of elongation activity ratios in muscle and backfat [212]. This may

explain higher composition of palmitic acid in Leghorn than broilers, since ELOVL6 was up-regulated in Leghorn.

Leghorn contained higher percentage of DHA, which is beneficial to humans by providing natural source of n-3 PUFA and also indicated the higher anti-inflammatory modulations in lean chickens. Dietary intake of n-3 PUFA reduced the incidence of myocardial infarction and chronic inflammatory or autoimmune disorders[213]. EPA and DHA reduce adiposity in humans and rodents either by increasing fatty acid oxidation in liver and adipose tissue or by inhibiting hepatic lipogenesis [214]. EPA and DHA also activates AMPK in adipose tissue and cultured adipocytes. Increased ratio of n-3 PUFA to n-6 PUFA in Fayoumi may be the reason for increased phosphorylated levels of AMPK in adipose tissue of Fayoumi chickens compared to broilers. DHA is synthesized through peroxisomal beta oxidation by shortening C24:6 n-3 [52]. An increase in DHA also implicated increased occurance of fatty acid oxidaiton in lean chickens compared to broilers.

Collectively, our results provided further evidence that the leanness of Leghorn and Fayoumi are associated with increased fatty acid oxidation in adipose tissue. Our results also provide an important basis to understand the potential connections between fasting, leanness and adipose inflammation, so that we can go further to investigate the relationship between nutritional status, fat deposition and the innate immune system in poultry.

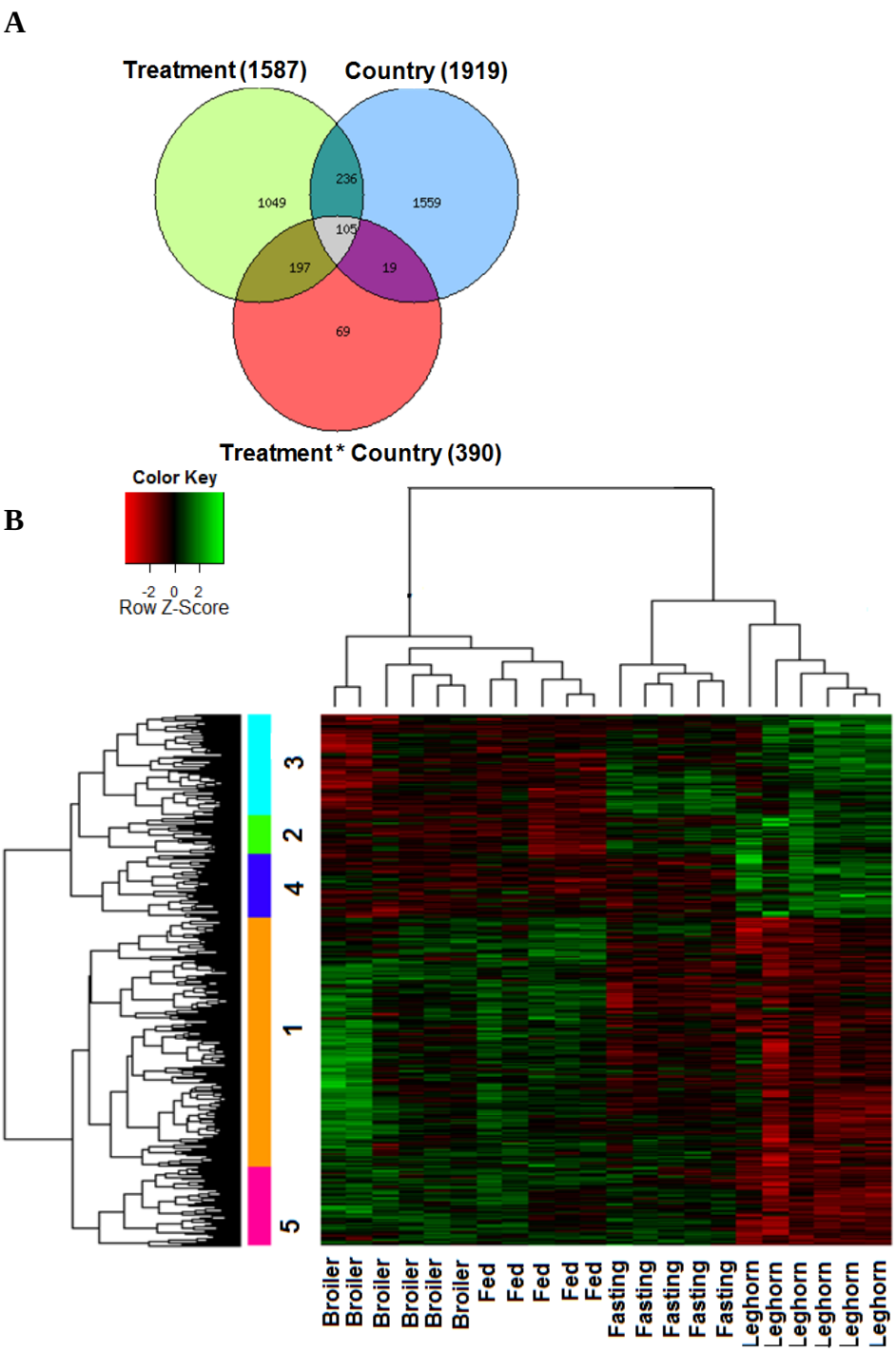


Figure 4-1. A. Venn diagram of differential expressed genes (adjusted pvalue<0.05) in terms of treatment factor, country factor and their interaction. B. Hierarchical cluster analysis of the 1049 genes (FDR adjusted pvalue <0.05) that were differentially expressed in terms of treatment. The colour bar denotes five clusters extracted by hierarchical cluster.

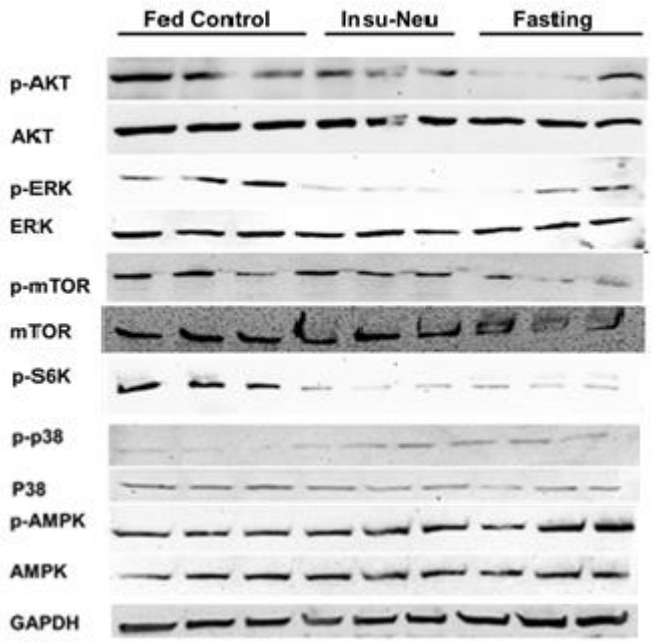


Figure 4-2. Western blots revealed increased abundance of phosphorylated p38MAPK and decreased phosphorylated ERK1/2 and S6K

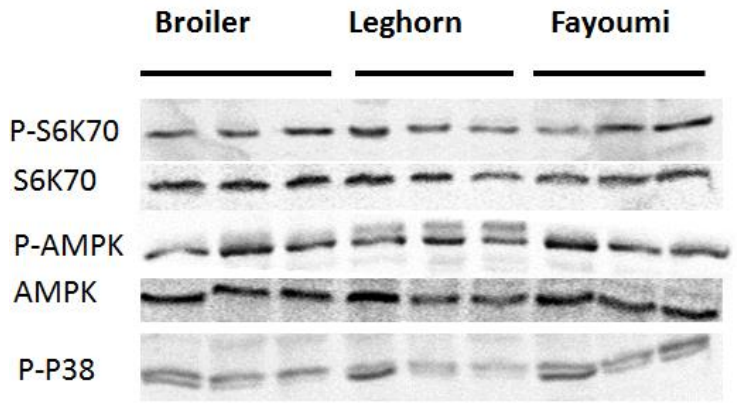


Figure 4-3. Western blots revealed increased abundance of phosphorylated p38MAPK in Fayoumi chickens

Table 4-1. Fatty acids composition in adipose tissue of broiler, Leghorn and Fayoumi

		Broiler	Leghorn	Fayoumi
myristic acid	14:00	0.0035[a]	0.0045[b]	0.0031[a]
myristoleic acid	14:1 (n-5)	0.0005[a]	0.0003[a]	0.0003[a]
palmitic acid	16:00	0.1672[a]	0.2168[b]	0.1445[a]
cis-7-hexadecenoic acid	16:1 (n-9)	0.0032[ab]	0.0026[a]	0.0029[a]
palmitoleic acid	16:1 (n-7)	0.0374[ab]	0.0213[a]	0.0245[a]
stearic acid	18:00	0.0392[a]	0.0669[b]	0.0429[ab]
oleic acid	18:1 (n-9)	0.4933[b]	0.3644[a]	0.5566[c]
cis-11-octadecenoic acid (cis-vaccenic)	18:1 (n-7)	0.0079[a]	0.0076[a]	0.0089[a]
linoleic acid	18:2 (n-6)	0.2426[a]	0.3113[b]	0.2124[a]
cis-5,8,11,14,17-Eicosapentaenoic acid (EPA)	20:5 (n-3)	0.0048[ab]	0.0040[b]	0.0038[a]
arachidonic acid (AA)	20:4 (n-6)	0.0002[ab]	0.0001[a]	0.0001[a]
all-cis-4,7,10,13,16,19-Docosahexaenoic acid (DHA)	22:6 (n-3)	0.0002[a]	0.0003[b]	0.0002[a]
	16:0/16:1	4.1214[a]	9.0468[b]	5.2659[a]
	18:0/(18:1+18:2)	0.0527[a]	0.0979[b]	0.0551[a]
	EPA+DHA/AA	27.9042[a]	45.5205[b]	60.4438[c]

Table 4- 2. Fatty acids composition in adipose tissue of broiler *ad libitum*, fasted for 5 hours and insulin-neutralized

		Fasting	Fed	Insu-Neu
myristic acid	14:00	0.0022	0.0021	0.0023
myristoleic acid	14:1 (n-5)	0.0002	0.0002	0.0003
palmitic acid	16:00	0.0704	0.0640	0.0687
cis-7-hexadecenoic acid	16:1 (n-9)	0.0033	0.0027	0.0031
palmitoleic acid	16:1 (n-7)	0.0218	0.0236	0.0213
stearic acid	18:00	0.0351	0.0320	0.0335
oleic acid	18:1 (n-9)	0.6708	0.6305	0.6187
cis-11-octadecenoic acid (cis-vaccenic)	18:1 (n-7)	0.0093[a]	0.0204[a]	0.0117[a]
linoleic acid	18:2 (n-6)	0.1838[a]	0.2213[a]	0.2378[a]
cis-5,8,11,14,17-Eicosapentaenoic acid (EPA)	20:5 (n-3)	0.0026	0.0026	0.0022
arachidonic acid (AHA)	20:4 (n-6)	0.0002	0.0002	0.0002
all-cis-4,7,10,13,16,19-Docosahexaenoic acid (DHA)	22:6 (n-3)	0.000278[ab]	0.000344[a]	0.000265[b]
	16:0/16:1	2.8091	2.4349	2.8144
	18:0/(18:1+18:2)	0.0516	0.0491	0.0532
	EPA+DHA/AA	16.1839	12.6882	15.9688

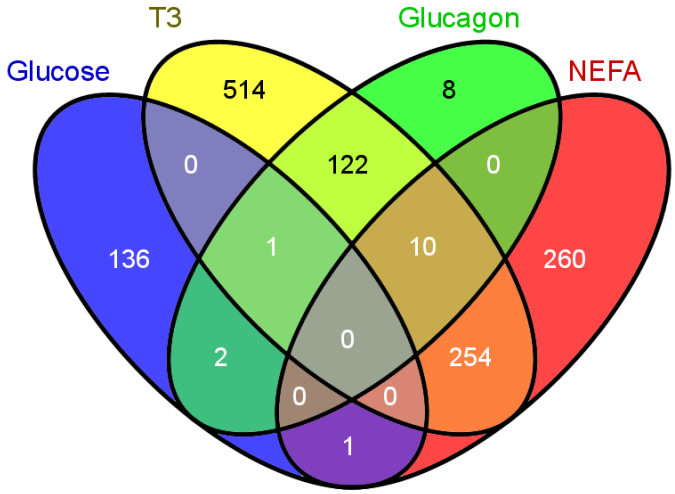


Figure 4-4. Four-way venn diagram of overlapping and unique genes highly correlated with plasma glucose, glucagon, NEFA, and T3.

CHAPTER V

CONCLUSIONS AND FUTURE PERSPECTIVES

We used an approach that couples comprehensive transcriptomic and metabolomic methods with system and cellular physiology to identify the mechanisms that regulate fat deposition in domestic chickens. The results are summarized as below:

A short term (5-hour) fast impacted expression of genes in a broad selection of pathways related to metabolism, signaling and adipogenesis. Fasting up-regulated rate-limiting genes in both the mitochondrial and peroxisomal pathways of beta-oxidation. Short term fasting suppressed adipogenesis; expression of key genes in multiple steps of adipogenesis, including lineage commitment from mesenchymal stem cells, were significantly down-regulated in fasted vs. fed adipose tissue. Microarray analysis of Fayoumi, Leghorn and broiler adipose tissue revealed that genetic leanness shared molecular signatures with the effects of fasting. Fatty acids components were significantly different between lean chickens and broilers. Chicken adipose tissue is refractory to insulin. Collectively, these data suggest that leanness in chickens is associated with increased fatty acid metabolism toward oxidation, rather than storage. It is surprising because white adipose tissue is not traditionally considered a site of significant fatty acid oxidation, which is more for muscle. Previous studies focused on fatty acid oxidation in liver and muscle, and ignored the occurrence of fatty acid oxidation in adipose tissue. Our study, for the first time, comprehensively investigated the association of adipose metabolic and physiological responses with gene expression in avian. Our study also identified the unique features of adipose metabolism of naturally lean chickens (layer and meat type) compared to broilers. These results also highlight

chicken as a useful model organism in which to study the dynamic relationship between food intake, metabolism, and adipose tissue biology.

Based on the findings of our studies, future studies will focus on reduction of fat deposition by regulation of lipolysis and fatty acid oxidation and the mechanism behind the effect of lipolysis and fatty acid oxidation. For example, does lipolysis induce activation of macrophages? Does overexpression of PDK4 in adipose tissue lead to increased fatty acid oxidation and reduced fatness? A tissue specific overexpression or knockout of PDK4 experiment is helpful to answer this question. Furthermore, how do fatty acid components dynamically change with lipolysis and fatty acid oxidation? It would also need to be determined if oxidative stress is associated with increased fatty acid oxidation.

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VITA

Bo Ji grew up in Laizhou, Shandong, China. She enrolled at Tianjin University of Science and Technology receiving her B.E. in Biological Engineering in 2002. She earned Master degree in Biology from Hong Kong Baptist University in 2006. After working in QIAGEN for three years, she came to the US in 2009 and pursued a PhD degree at University of Tennessee Knoxville. Her Ph.D. degree in Animal Science and Master degree in Statistics were conferred in May of 2013.