MOLECULAR BASIS OF FEED EFFICIENCY IN MEAT-TYPE CHICKENS

by

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(Under the Direction of Dr. Samuel E. Aggrey)

ABSTRACT

In poultry production, feed accounts for 50 to 70% of total cost in raising a chicken. Feed cost has increased dramatically in recent years and decreasing the amount of feed per unit of weight gain will improve efficiency of production and increase profits. The improvement of feed efficiency in chickens is lagging behind other traits such as growth and body composition. In addition, our understanding of genes that affect feed efficiency in chickens is inadequate. The explosion in molecular technology techniques has made it possible to delineate the molecular basis of feed efficiency. Genetic markers in the genes that affect feed efficiency can be developed to assist in conventional selection strategies. The general goal of this thesis is to study molecular and biological functions of genes that underlie feed efficiency and nitrogen recycling in a chicken population divergently selected for feed efficiency. Gene networks associated with residual feed intake (RFI) through transcriptional profiling of duodenum at two different ages in a chicken population divergently selected for low or high RFI were identified. The genes that were differentially expressed between chicken lines with low and hign RFI are functionally associated with residual feed intake and genetic markers can be developed in those genes to aid selection for genetic improvement.

INDEX WORDS: Chickens, transcriptomics, feed efficiency, nitrogen excretion, mTOR

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CHAPTER 1

Introduction

Poultry has been an important protein source for humans for the past 50 years. In US per capita consumption of broiler meat increased from 24.5 to 36.3 kg between 2000 and 2009. In poultry production, feed accounts for 50 to 70% of total cost in raising a chicken. Moreover, because feed cost has increased dramatically in recent years, decreasing the amount of feed per unit of weight gain will improve efficiency of production and increase profits. Today's poultry production has grown in both meat and egg production through selective breeding. Especially, rapid growth and enhanced muscle mass of chickens are the result of selection over many generation. However, feed efficiency of chickens has not caught up with other traits such as growth and body composition. The reasons for lack of sufficient improvement in feed efficiency trait can be explained by inconsistent selection goals, a lack of consistent definitions of efficiency, and focus on population similarities rather than individual variation (Johnson et al., 2003). Therefore in order to improve feed efficiency, new approaches based on molecular genetics, such as global gene expression microarray and single nucleotide polymorphism (SNP) are required to derive feed efficiency genotypes for genetic improvement.

Currently there is a poor understanding of genes that affect feed efficiency in chickens. Explosion in molecular technology and bioinformatic computational strategies have made it possible to delineate the molecular basis of feed efficiency. Genetic markers in the genes that affect feed efficiency can be developed to assist in conventional selection strategies.

The general goal of this thesis is to study molecular and biological functions of genes that underlie feed efficiency and nitrogen recycling in a chicken population divergently selected for feed efficiency. The specific objectives are:

1. To perform global gene expression study using microarray and real-time reverse transcription polymerase chain reaction (RT-PCR) in a chicken population divergently selected for residual feed intake.
2. To study the gene expression differences in genes in the avian mechanistic target of rapamycin (mTOR) pathway and how they affect feed efficiency.
3. To investigate the gene expression differences in genes in the nitrogen recycling pathway in a chicken population divergently selected for residual feed intake.

This research will contribute to understanding the molecular mechanism of feed efficiency in chickens through transcription profiling of many genes including potential targets such as SNPs to breed chickens with high feed efficiency trait. In addition, this approach can be opportunities to explain physiological complications associated with genetic selection for feed efficiency.

CHAPTER 2

Literature review

Definitions of Feed efficiency

The feed efficiency is an interaction between feed intake and body weight gain. Feed intake cannot be isolated from output trait; therefore it is not possible to measure production system efficiency within a single production system. Several definitions of feed efficiency have been proposed as many investigators use different parameters for measurement. Some of the notable definitions are summarized in Table 2.1.

Feed conversion ratio (FCR; gross efficiency)

The index of feed efficiency which has been used widely is feed conversion ratio (FCR). It is also called as gross efficiency. FCR is defined as the ratio between feed inputs and production outputs. In meat-type livestock, feed efficiency is generally expressed as the ratio between feed intake and weight gain measured over a defined period of growth. Research for FCR began 60 years ago. Hess et al. (1941) first demonstrated that there was a heritable difference in feed efficiency between individuals and the difference of feed efficiency could be distinguished from that of body weight, body weight gain, or age. Marks (1991) showed there was a big difference (36%) in feed efficiency between selected and unselected lines during first two days after hatch when the difference in body weight was small but the difference of feed efficiency decreased significantly by day 14. It means that increases of age changes body weights between selected and unselected and different weights cause different maintenance

requirements. Thus, the different maintenance requirement somehow limits feed efficiency concept from reflecting feed for growth. As the result, the increase of body weight with age as well as age-constant FCR could be considered.

Chambers and Lin (1988) suggested weight-constant measures for feed efficiency. They showed negative correlation (-0.5 to -0.8) of body weight and body weight gain with feed intake in weight constant measures and positive correlation (0.5 to 0.8) in age constant measures. Age-constant and weight-constant feed efficiency had high positive correlation (0.8 to 0.9). Thus, they concluded that age-constant and weight-constant feed efficiency could be applicable for reflection of true genetic feed efficiency. Atchley et al. (2000) also explained that statistically, FCR is a ratio trait is not normally distributed, without any real mean and variance. When coefficient of variation of the denominator increases, non-normality of a ratio trait would be increase. The methods base on ratio values are often difficult to measure on the individual and have problems inherent in selection by ratio measures (Gunsett, 1984). Furthermore, FCR improved by strong selection will produce greater maintenance energy costs and in turn, cause to increase mature sizes (Taylor, 1985).

Maintenance efficiency

Maintenance requirement is an important factor to determine the efficiency of production output. Ferrell and Jenkins (1985) defined maintenance requirement as the feed energy required for zero body weight change after allowing for different energy densities of body components. Thus, maintenance efficiency can be defined as the ratio of body weight to feed intake at zero body weight change. However, it is difficult to measure the maintenance efficiency practically. Because the requirement for weight stasis could not be satisfied in growing animal,

the application of the maintenance efficiency associated with measurement has limitations. It is also necessary to hold animals at constant weight to measure true maintenance requirements and in the case of beef cattle, it could take up to 2 years (Taylor et al., 1981).

Partial efficiency of growth

Geay and Micol (1988) defined partial efficiency of growth as the ratio of weight gain to feed intake after expected maintenance feed requirements are subtracted. The maintenance requirement can be estimated from feeding tables (e.g., SCA, 1990) based on average body weight during the measurement period or metabolic studies of the energy balance of animals (ARC, 1980). However, the methods have problems in terms of their measurement.

Residual feed intake

Koch et al., (1963) suggested the concept of residual feed intake (RFI) as an alternative method to measure feed efficiency. In their research, it was suggested that feed intake could be adjusted for body weight and weight gain. Thus, feed intake could be partitioned into the feed intake expected for the given level of production and a residual part. The residual part of feed intake can be deviated from their expected level of feed intake. That is, RFI can be defined as feed intake of the expected feed requirements for maintenance and growth, with expected feed requirements obtained by regression. RFI value is independent on the production traits because it uses feed intake values. Thus, the advantage of use of RFI is that it allows comparison between individuals differing in level of production during the measurement period.

Genetic parameters of residual feed intake have been published since 1960's. RFI is generally heritable in growing and lactating cattle ranging from 0.16 to 0.39 (Koch et al., 1963;

van Arendonk et al., 1991; Jensen et al., 1992; Ngwerume and Mao, 1992; Arthur et al., 2001a; Nkrumah et al., 2007), swine 0.23 to 0.38 (Foster et al., 1983; Mrode and Kennedy, 1993; Cai et al., 2008), and laying hens 0.20 to 0.50 (Wing and Nordskog, 1982; Bentsen, 1983; Luiting and Urff, 1991; Bordas et al., 1992; Shulman et al., 1994). However, only a few studies for RFI have been reported in broilers 0.21 to 0.49 (Van Bebber et al., 1994; Pakdel et al., 2005; Aggrey et al., 2010). Selection for low RFI leads to the reduction of feed consumption without inhibited production and ultimately brings improved feed efficiency. As the reason, many researches across species have demonstrated that they still have similar BW and production performance although animals selected for RFI showed great differences in feed intake (Cai et al., 2008, Arthur et al., 2001b). The genetic correlations between RFI and feed efficiency or FCR are generally range from 0.60 to 0.75 in livestock species (Arthur et al., 2001a and b; Cai et al., 2008) and the correlation between RFI and BWG (body weight gain) was ranged from near zero to positive (Arthur et al., 2001a and b; Pakdel et al., 2005; Aggrey et al., 2010).

Physiological basis for variation in feed efficiency

Feed consumed by a livestock is generally portioned into required maintenance and production. Maintenance requirements include maintaining basal metabolism, performing body activity, and producing the heat for keeping body temperature (Korver, 1988). Thus, RFI reflects variation sources of biological processes in terms of feed efficiency such as differences in requirements for maintenance, body composition, proportions of visceral organs, physical activity and digestion efficiency (Archer et al., 1999). Luiting and Urff (1991) suggested that activity-related expenditures account for 30 to 50% of the difference in total energy expenditures among birds with divergent RFI and differences in basal metabolism or heat increment can

explain the remaining 50 to 70%. It indicates that the variation in RFI was generally related to differences in maintenance energy requirements. Herd et al. (2004) found that one-third of the biological variation in RFI of growing beef calves could be due to interanimal differences in digestion, heat increment, composition of gain and activity, whereas the remaining two-thirds of variation in RFI may be related to interanimal differences in energy expenditures associated with biological processes such as ion pumping (i.e., Na^+/K^+ ATPase), mitochondrial proton leak, uncoupling proteins (UCP), thyroid hormones, leptin, IGF-I, lipid metabolism enzymes, or sympathetic activity (Johnson et al., 2003).

RFI was positively correlated ($r_p = 0.56$) with heat production (Basarab et al., 2003) and maintenance energy requirements $r_p = 0.42$ (Castro Bulle et al., 2007) in growing steers. Steers with low RFI had 21% less heat, 28% less methane energy losses, and 11% greater digestible energy compared to high RFI (Nkrumah et al., 2006). Also, calves with low RFI showed greater total digestibility (Richardson et al., 1998; Krueger et al., 2008). However, there was no difference in total digestibility between poultry or pigs with divergent RFI (Luiting et al., 1991; De Haer et al., 1993). Physiological processes related to interanimal variation in energy expenditures, mitochondrial proton leak, Na^+/K^+ ATPase, and protein turnover each accounts for approximately 20% to the total interanimal variation in basal energy expenditures (Brand, 1990; Rolfe and Brand, 1997). Maintenance energy requirements were phenotypically correlated ($r_p = 0.76$) with protein degradation rates in steers (Castro Bulle et al., 2007). In addition, thyroid hormone mechanisms have been shown to regulate dietary-induced thermogenesis (Gabarrou et al., 1997a). Gabarrou et al. (1997b) found that the hepatic deiodinase conversion of T_4 to T_3 with iopanoic acid was inhibited in high RFI cockerels compared to low RFI phenotypes and it mediated the increase of heat increment in energy expenditures.

Mitochondrial function and feed efficiency

The most important role of mitochondria is to generate 90% of cellular ATP through respiration and to regulate cellular metabolism. The central set of reactions involved in ATP production is known as the citric acid cycle, or the krebs cycle. However, the mitochondria have many other functions in addition to the production of ATP. Mitochondria have a electron transport chain (ETC) which couples a reaction between an electron donor (such as NADH) and an electron acceptor (such as O_2) to the transfer of H^+ ions across a membrane. Finally mitochondria produce H_2O_2 and cause a proton motive force providing energy for ATP synthesis.

Assessments of mitochondria function

In assessing mitochondria function, there are three methods to monitor O_2 consumption such as respiratory control ratio (RCR) and ADP to oxygen ratio (ADP: O). RCR (state III respiration/state IV respiration) is an index of respiratory chain coupling and it indicates the efficiency of coupling of electron transport (Estabrook, 1967). ADP: O indicates the efficiency of ATP synthesis coupled to cell respiration, that is, the ability to carry out oxidative phosphorylation (OXPHOS). It is calculated by dividing the nanomoles of ADP by the amount of O_2 consumed (Estabrook, 1967).

Another assessment is reactive oxygen species (ROS). During normal cellular metabolism, mitochondrial electron transport results in the formation of superoxide anion (O_2•–) and subsequently hydrogen peroxide (H_2O_2). Because H_2O_2 increases in concentration under certain physiologic and pathophysiologic conditions and can oxidatively modify cellular components, it is critical to understand the response of mitochondria to H_2O_2. Generally, by

measuring the H_2O_2 production, ROS can be assessed. That is, ROS is index of H_2O_2 production; As H_2O_2 increases, ROS increases. High H_2O_2 production means elevated electron leak and site-specific defects in electron transport.

Feed efficiency and mitochondria function

Previous studies have explained that the low feed efficiency phenotype generally has low ETC coupling efficiency and site specific defects due to ROS-mediated oxidation (e.g. increase H_2O_2; Sohal, 1993; Petrosillo et al., 2003; Ferguson et al., 2005; Bottje et al., 2002). Thus, Bottje et al. (2002) hypothesized that in low feed efficiency compared with high feed efficiency mitochondria, the ROS-mediated oxidation caused low feed efficiency mitochondria to have less complex activities and a general decrease in all complex activities (with one exception in complex IV of the duodenum). The less complex activities associated with elevated ROS production Indicate that decreases in complex activities in low feed efficiency can be due to ROS-mediated damage to the protein complexes (Bottje et al., 2009)

Oxidative Stress and Protein Oxidation

The glutathione (GSH) redox system is a vital defense mechanism of mitochondria because mitochondria lack the ability to synthesize GSH (Meister, 1984), and to export oxidized GSH (GSSG) against free radical damage (Olafsdottir and Reed, 1988; Bottje et al., 2009). Thus, the GSSG to GSH ratio is used as an indicator of oxidative stress (Kidd, 1997) because reduced GSH is important in protecting the ETC from oxidation (Bottje et al., 2009). That is, the GGSG to GSH ratio indicates the presence of an oxidative stress. Ojano-Dirain et al. (2005a) demonstrated more oxidative stress in duodenal mitochondria from low feed efficiency broilers

suggesting greater GSSG to GSH ratio. It might mean that low feed efficiency broilers would experience greater oxidative stress compared with high feed efficiency broilers.

One of major indicators of protein oxidation is protein carbonyls (Stadtman and Levine, 2000). In low feed efficiency birds, from Breast muscle mitochondria, as well as gut, leg, heart, liver, and lymphocyte homogenates, greater protein carbonyls were observed compared with high feed efficiency birds. This result concurs with previous studies suggesting greater oxidative stress in low feed efficiency mitochondria.

Genes related to mitochondria function in feed efficiency

Briefly, many studies for mitochondria function in feed efficiency suggested that the phenotype of low feed efficiency chickens could be due to the inefficient mitochondrial function. The expression patterns of the genes involved in mitochondrial energy metabolism - avian adenine nucleotide translocator (avANT), cytochrome oxidase III (COX III), avian uncoupling protein (avUCP), inducible nitric oxide synthase (iNOS), peroxisome proliferator-activated receptor-γ (PPAR-γ), avian PPAR-γ coactivator-1α (avPGC-1α) - in breast muscle and duodenum from low and high feed efficiency broilers have been investigated (Ojano-Dirain et al., 2007b). These studies assume that increased ROS in low feed efficiency phenotype may change the expression of above genes.

COX III

Because COX III is responsible for regulating proton pumping and electron transport (Scheffler, 1999), COX III might have the function to control energy balance. Several studies demonstrated that COXIII was expressed differently with broiler feed efficiency phenotype from

several tissues (Iqbal et al., 2004 and 2005; Lassiter et al, 2006; Ojano-Dirain et al., 2005a and b) However, Ojano-Dirain et al. (2007b) showed that the COXIII mRNA levels were not different between the duodenum of high and low feed efficiency birds.

avANT

ANT is responsible for the exchange of cytosolic adenosine diphosphate for mitochondrial matrix ATP across the inner mitochondrial membrane. Mammalian ANT has 3 isoforms: ANT-1, ANT-2, and ANT-3. The ANT-1 has been known as a metabolite to mediate mitochondrial energy production (Malgat et al., 2000). That is, the higher expression of ANT-1 in tissues indicates higher energy demand by an animal. Thus, low feed efficiency line with higher energy demand is expected to have higher ANT-1 expression compared with high feed efficiency line. However, conflicted researches have been reported. Although greater ANT-1 expressed in the low feed efficiency breast muscle (Iqbal et al., 2004), Ojano-Dirain et al. (2005b and 2007b) found that breast muscle avANT mRNA levels were less in the low feed efficiency broilers. On the other hand, on protein levels, the ANT protein in broilers was expressed highly in the low feed efficiency breast muscle (Iqbal et al., 2004) but not different between feed efficiency groups in the liver (Iqbal et al., 2005) and duodenum (Ojano-Dirain et al., 2005b).

UCP and iNOS

UCP is known as a facilitator of proton leak across the inner mitochondrial membrane, which is responsible for 20 to 30 % of basal metabolism oxygen consumption (Brand, 1990). Raimbault et al. (2001) reported that leg muscle from low feed efficiency chickens (Bordas and Mérat, 1984) had higher avUCP mRNA expression than that from high feed efficiency line.

Moreover, Bottje et al. (2006) found higher avUCP mRNA expression in breast muscle from of high feed efficiency birds. It has been known that ROS and nitric oxide (NO) are linked to oxidative stress of mitochondria. In increased mitochodirial oxidative stress by ischemia and hypoxia, the production of ROS and NO increased (Schild et al., 2003; Chen et al., 2001). NO is synthesized from L-argiine by NO synthase (NOS). NOS is present in 3 isoforms: neuronal NOS and endothelial, and inducible (iNOS). In mouse, ablation of iNOS in ob/ob mice caused decreased feed intake and developed energy efficiency ultimately (Becerril et al., 2010). In chicken duodenum but not breast, iNOS was expressed more highly in the high feed efficiency line than in the low feed efficiency line (Ojano-Dirain et al., 2007b).

PPAR and PGC 1-α

PPAR belongs to a family of ligand-activated transcription factors that control key metabolic pathways (i.e., adipogenesis, fat metabolism, and insulin signaling; Lee et al., 2003). Nisoli et al. (2003) provided evidence that differences in mitochondrial protein expression may be due to nuclear regulation to include induction of PPAR-γ and PGC-1α. In duodenum, PPAR-γ was expressed lowly in the low feed efficiency birds but there was no difference of avPGC-1α between the high and low feed efficiency lines (Ojano-Dirain et al., 2007b). In breast muscle, the two genes had no differences of their expression between the two lines (Ojano-Dirain et al., 2007b). On the other hand, in hepatic tissues, PPAR-γ expression increased after hatching and refeeding and in the chickens selected for rapid growth (Cogburn et al., 2007).Moreover, many other proteins expressed differently between HFE and LFE broilers have been reported. For example, collapsin-2, part of a group of semiphorin proteins (Adams et al., 1997), was greater in lymphocytes of low feed efficiency compared with high feed efficiency broilers (Lassiter et al.,

2006). Vinculin (a focal adhesion regulator involved in cell-cell communication), plasma transthyretin (a thyroid hormone and retinol transport protein), histone deacetylase (involved in gene regulation), and adophilin (associated with lipid accumulation) were expressed differently at least 3-fold between feed efficiency phenotype groups (Bottje et al., 2009)

Thus, transcription factors, other genes encoding mitochondrial proteins and proteins associated with mitochondria function may be critical determinants related to phenotypic expression of feed efficiency in broilers.

Central nervous system regulation of energy expenditure and body weight

Energy balance and body weight are influenced by autonomatic nervous system which controls energy balance neuroendocrine system under autonomic nervous system. Energy intake is the most important factor in feed efficiency and energy intake is controlled directly by feeding behavior. Regulation of feeding behavior may be explained two systems, short- and long-term regulation of energy balance (Richards et al., 2003). A short-term regulation can be referred to as "peripheral satiety system". That is, short term control is meal-related signals, which affect initiation and termination of meals. The short-term control of feed intake includes both hormonal and neural signals. These satiety signals in short-term control are stimulated by the presence of feed in the gastrointestinal tract, the pancreas and liver as well as the gut. These are transmitted to the brainstem via the activation of neural (vagal) afferent pathways or via secretion of signaling substances into the bloodstream (Jensen, 2001, Richards et al., 2003).

A long-term regulation system provides information about the amount of energy stores (e.g., adipose tissue mass) to the hypothalamus. Thus, Long-term regulation of energy balance occurs via neural and neuroendocrine pathways activated in the hypothalamus integrating with

satiety signaling pathways originating in the brainstem. By cumulative regulation of meal-related signals (short-term regulation) with long-term maintenance of energy storage, long-term system achieves energy homeostasis and, ultimately, promotes maintenance of body weight (Woods et al., 1998; McMinn et al., 2000; Jensen, 2001; Blevins et al., 2002; Berthoud, 2002; Richard, 2003).

The control of feed intake and energy expenditure leads to homeostasis of body energy store meeting immediate energy demands. The regulation of feed intake and energy homeostasis were regulated by multiple peptidergic neuronal pathways. These pathways can be involved in two basic categories, anabolic and catabolic (Woods et al., 1998). Anabolic and catabolic pathways result in a net increase and decrease, in energy intake and storage respectively.

Satiety signals

There are two types of satiety signals: ghrelin which stimulates feeding behavior and cholecystokinin (CCK) which inhibits it (Woods et al., 1998; McMinn et al., 2000; Jensen, 2001; Blevins et al., 2002). The examples of both types of satiety signals have been reported in poultry species. Ghrelin has the function as a releasing factor for growth hormone (GH) through the GH secretagogue as well as a controller feeding behavior (Furuse et al., 2001; Ahmed and Harvey, 2002; Kaiya et al., 2002; Saito et al., 2002). However, contradictory effects of ghrelin were shown that it inhibits feed intake when administered centrally to chickens (Furuse et al., 2001; Saito et al., 2002). CCK has been known as a potent inhibitor of feeding in birds (Denbow, 1994; Kuenzel, 1994; Jensen, 2001). CCK functions as a satiety signal to depress appetite to the brainstem and delays gastric emptying.

Hormonal regulation of feed intake

Leptin and insulin

Leptin and insulin are major hormonal signals from the hypothalamus to be involved in anabolic and catabolic pathways in mammals (Richards et al., 2003). In mammal, leptin is known to inhibit feed intake. However, in chicken, it is still controversial whether the leptin gene really exists because some researchers failed to reproduce the cloning of chicken leptin gene (Friedman-Einat et al., 1999). However, the effects of exogenous (human as well as ‘‘chicken/mouse like”) recombinant leptin on chicken cell culture (Shi et al., 2006, Denbow et al., 2000; Taouis et al., 2001) have been published and chicken leptin receptor genes have been cloned (Horev et al., 2000; Ohkubo et al., 2000). Thus, in chickens, energy store and expenditure could be controlled by different physiological mechanism from mammals.

On the other hands, insulin receptors have been identified in the brain tissues of birds (Simon and Leroith, 1986). Sonoda (1983) reported that the circulating insulin level was low before feeding and it increased after feeding in chickens. In detail, plasma levels of leptin and insulin fall and rise with decrease and increase in energy state in chickens such as during fasting and refeeding, respectively (Richards et al., 2007). It ultimately suggests that these two key hormones, leptin and insulin could serve as peripheral signals of energy state in birds.

Other hormonal peptides

In mammals, neuropeptide Y (NPY) is known to stimulate feed intake (Edwards et al., 1999). The major site of expression for NPY within neuron in hypothalamus is hypothalamic arcuate nucleus (ARC). Because the ARC is the site to project to the paraventricular nucleus, the ventromedial nucleus and other sites, NPY expression in the ARC may indicate interaction of

NPY with other orexigenic and anorexigenic signals in the hypothalamus (Sahu et al., 1998). Central administration of NPY stimulated increase in feed intake in rats (Clark et al., 1984). In chickens, the NPY gene has been identified and was suggested to be localized in the brain (Blomqvist et al., 1992; Wang et al., 2001a). It also has been reported that NPY gene expression in the brain changes in energy status induced by fasting and feed restriction (Boswell et al., 1999). Futhremore, Kuenzel and McMurtry (1988) revealed that NPY functions as a potent orexigenic agent when administered centrally in chicken. Specific NPY receptors (Y1 and Y5) also have been found to mediate NPY effects on feeding behavior in chickens (Holmberg et al., 2002).

Glucagon is one of the peripheral peptide hormones and generally has been known to be involved in energy homeostasis. In rats and chickens, central administration of glucagon leaded to suppression of feed intake (Inokuchi et al, 2007). Glucagon superfamily may be divided two major branches: a) glucagon; the glucagon-like peptide-1 (GLP-1), GLP-2 and b) growth hormone releasing factor (GRF); pituitary adenylate cyclase-activating polypeptide (PACAP); vasoactive intestinal peptide (VIP). Among the members of glucagon superfamily, the glucagon-like peptide-1 (GLP-1) is known well to have an important role on feeding behavior because the GLP-1 receptor binds only its own ligand and does not bind other glucagon members. Thus, it has been shown that central administration of GLP-1 suppressed feed intake in rats (Honda et al, 2007). In chickens, GLP-1(Tachibana et al., 2004), pituitary adenylate cyclase-activating polypeptide (PACAP) and VIP (Tachibana et al., 2006) have been known for their function to inhibit food intake. In addition, growth hormone releasing factor (GRF) was reported to suppress feed intake in neonatal chicks (Furuse et al,. 2001). Based on these results, the glucagon superfamily can be suggested to control satiety signal of chicks as an anorexigenic factor.

Thachibana et al. (2003) compared the effect of glucagon superfamily. Intracerebroventricular injections (ICV) of VIP, PACAP, GRF and GFP-1 showed that the anorexigenic effect of GRF and GFP-1 were stronger than that of VIP and PACAP.

Corticotropin-releasing factor (CRF) plays multiple roles in endocrine systems (Benoit et al, 2000). CRF is also thought to be related to regulate of feed intake. In mammals, administration of CRF inhibited feed intake (Benoit et al., 2000, Contarino et al., 2000, Hope et al., 2000). Urocortin 1 and urotension have been known to have biological homology of CRF family (Britton et al., 1984, koob 1999). Zang et al. (2001) compared suppressive effects of feed intake for the CRF family members. Food intake decreased more strongly by CRF followed by urotensin I, and then, urocortin. ICV injection of Histamine also has been shown to inhibit feed intake in rats (Lechklin et al, 1998). In chickens, histamine significantly suppressed food and water intake in a dose-dependent manner, which is mediated by both histamin-1 and -2 receptors (Meade and Denbow, 2001).

Bombesin is originally isolated from the skin of the oriental fire-bellied toad (Bombina orientalis). In mammals, two homologs of Bombesin have been known as neuromedin B and gastrin-releasing peptide. Bombesin stimulates gastrin release from G cells and is one of major anorexigenic factors in mammals (Gilbbs et al, 1979). In chickens, Bungo et al. (2000) suggested that ICV injection of Bombesin decreased feed intake of neonatal chicks following dose dependent manner. Bungo et al. (2000) also investigated the interaction of Bombesin with NPY. In the study, increased feed intake by central administration of NPY was inhibited by injection of Bombesin. This results shows that bombesin may interact with NPY for control of feed intake. Moreover, L-pipecolic acid (L-PA) is a cyclic imino acid produced during the degradation of lysine. It has been demonstrated that ICV injected L-PA leaded to decrease food intake in the

neonatal chicks (Takagi et al., 2001). Previously Takahama et al. (1986) suggested the influence of L-PA on ϒ-aminobutyric acid (GABA) response in the rat brain. L-PA inhibited the cellular uptake of GABA in the brain. Thus, Takagi et al. (2003) investigated a possible relationship between L-PA and GABA in chickens. However, although L-PA activates both GABA-A and GABA-B receptors, only GABA-B receptors affects on food intake.

Hypothalamic regulation of feed intake- Melanocortin pathway

POMC and AGRP

The best characterized neuronal pathway involved in the regulation of feed intake and energy expenditure is the central melancortin system. It includes neurons that express proopiomelanocortin (POMC), a precursor containing α-Melanocyte stimulating hormone and a second set that express NPY and agouti-related peptide (AGRP; Cone, 2005).

POMC is a precursor for the melanocortin peptides, α-, β-, and γ-melanocyte stimulating hormone (MSH) and the endogenous opiod, β-endorphin. Five melanocortin receptors (MC1R, MC2R, MC3R, MC4R, and MC5R) have been characterized (Moutntjoy et al., 1992; Adan and Gispen 2000). α-MSH is known as an inhibitor of food intake in rats and chickens (Ludwig et al. 1998; Kawakami et al., 2000). In detail, central administration of α-MSH significantly suppressed feed intake of 3h fasted neonatal chicks in a dose-dependent manner (Kawakami et al., 2000b). ICV injection of α-MSH or a synthetic melanocortin agonist for MC3R and MC4R suppressed feed intake in mammals suggesting potential roles of MC3R and MC4R in the regulation of feed intake (Poggioli et al., 1986; Fan et al., 1997; Rossi et al., 1998; Murphy et al., 2000; Wirth et al., 2001). In chickens, POMC gene has been cloned and showed the same structural organization as that of other species (Takeuchi et al., 1999; Gerets et al., 2000;

Kawakami et al., 2000). However, although in mammals, MC3R and MC4R showed high expression in brain tissues (Granz et al., 1993), MC3R was not expressed in the RT-PCR analysis using chicken brains (Takeuchi and Tkahashi 1999).

AGRP is synthesized in the hypothalamic arcuate nucleus (ARC). In rats and mice, ICV injection of AGRP stimulated feed intake (Hagan et al., 2000, Kim et al., 2000, Lu et al, 2001) and AGRP suppressed induced feed intake by α-MSH and a synthetic agonist for MC3R and MC4R (Rossi et al., 1998). In addition, over expression of AGRP by genetic manipulation resulted in obesity of mammals (Ollmann et al., 1997, Marks and Cone 2001). In chickens, the AGRP gene homologue has been identified and sequenced (Takeuchi et al., 2000). As it does in mammals, AGRP has been found to serves as an antagonist of α-MSH in chickens by binding to specific melanocortin receptor subtypes (MC3-R and MC4-R). When administered intracerebroventricularly, AGRP was orexigenic in layer chickens, but not broilers (Tachibana et al., 2001). The majority of neurons that express AGRP also coexpressed with NPY indicating a possible interact of AGRP with NPYin mammals and chickens.

Relationship between the components in melanocortin system

The neurons expressing NPY/AGRP are categolized as anabolic pathway whereas the POMC-expressing neurons are categolized as catabolic pathways. Briefly, negative energy balance (e.g., a fasting condition) from peripheries to the ARC suppresses the POMC catabolic pathway and induces the NPY/AGRP anabolic pathway. Thus, it ultimately leads to increased feed intake. On the other hand, positive energy balance suppresses the NPY/AGRP and stimulates the POMC anabolic pathway. Thus, it leads to decreased feed intake. The balance in

the activity of these two circuits within the hypothalamic melanocortin system controls energy status.

Nutrient and energy metabolic signals

AMPK pathway

One of mechanism to regulate whole body energy balance is nutrient (fuel) and metabolite sensing pathways within hypothalamic neurons. As such an example, adenosine monophosphate-activated protein kinase (AMPK), is a serine/threonine kinase and a central component of a kinase-signaling cascade that plays a critical role in maintaining cellular energy homeostasis (Hardie, 2004; Carling, 2005; Kahn et al., 2005; Hardie et al., 2006). AMPK is also an energy sensing enzyme. Metabolic and environmental stresses that deplete cells of energy (adenosine triphosphate) activate AMPK. Activation of AMPK inhibits ATP-consuming anabolic pathways and stimulates ATP-producing catabolic pathways in an attempt to restore cellular energy (Richards et al., 2007). In detail, AMPK stimulates glucose uptake, glycolysis, fatty acid oxidation, and mitochondrial biogenesis and inhibits protein synthesis, glycogen synthesis, glucogenesis, and fatty acid/cholesterol synthesis.

In terms of hypothalamic neurons regulated by AMPK, Richard et al. (2007) suggested the malonyl-CoA hypothesis. The AMPK inhibits the activity of acetyl-CoA carboxylase which catalyzes the conversion of acetyl-Co A to malonyl-CoA. The AMPK also stimulates the activity of malonyl-CoA decarboxylase which induces the conversion of malonyl-CoA to acetyl-CoA. Thus, the AMPK reduces the levels of two key metabolites, malonyl-CoA, and long chain fatty acyl-CoA. The increase of these metabolites' levels indicates an elevated energy status and depresses FI and BW gain (Dowell et al., 2005). That is, the key metabolites (malonyl-CoA and

long chain fatty acyl-CoAs) serve as signals of energy status and induce reduced expression of orexigenic neuropeptides (NPY/AGRP) and increased expression of anorexigenic neuropeptides (POMC) within hypothalamic neurons (Hu et al., 2003; Lane et al., 2005) that control feed intake and peripheral energy expenditure.

In addition, the stimulation of AMPK is depressed by the hormones, leptin and insulin in the mammal hypothalamus (Minokoshi et al., 2004; Carling, 2005). As the result, leptin and insulin stimulates increased activity of acetyl-CoA carboxylase. The elevated acetyl-CoA carboxylase leads to an increased level of malonyl-CoA, which promotes anorexigenic signaling. Finally, anorexigenic signaling causes decreased body weight through reduced feed intake and increased energy expenditure (Wolfgang and Lane, 2006). In chickens, recently genes related to AMPK pathway have been reported through the identification of genes for upstream AMPK kinases, LKB1 and calcium/calmodulin dependent protein kinase (Proszkowiec-Weglarz et al., 2006b). The functional AMPK pathway in birds with similar characteristics to mammals was also demonstrated by detection of active (phosphorylated) AMPK in peripheral tissues as well as in hypothalamic feeding centers (Proszkowiec-Weglarz et al., 2006).

mTOR pathway

Mammalian target of rapamycin (mTOR) has been proposed as a new hypothalamic fuel-sensing/signaling pathway. mTOR has its function as a sensor of energy status which regulates feed intake and energy balance (Cota et al., 2006). In detail, mTOR acts as an ATP sensor because elevated cellular ATP increases mTOR activity. Thus, this signaling pathway can induce depressed feed intake and BW through increasing energy expenditure (Cota et al., 2006). Furthermore, in mammals, it has been reported that hypothalamic mTOR activity is regulated by

leptin and insulin. Leptin regulates energy store and expenditure, and is controlled by insulin (Havela et al., 2000). Leptin stimulates mTOR activity via the signal transducer STAT3, a product of signaling through the leptin receptor (Cota et al., 2006). However, in chickens, because it has been shown that the leptin gene may not exist (Friedman-Einat et al., 1999), chickens may have a different physiological mechanism related to mTOR from mammals for control of energy store and expenditure. In addition, Akt/TOR/p70S6K pathway was characterized in chickens (Duchene et al., 2008a) and the activity of Akt, TOR, 4E-BP1, p70S6K (known as S6K1) and S6 regulated by refeeding and insulin treatment in muscle tissues was suggested. Duchene et al. (2008b) demonstrated that S6K1 activity regulated by insulin was different between muscle tissues of fast- and slow-growing chickens. Insulin in muscles affects S6K1 activity more strongly in the fast-growing chickens than in the slow-growing chickens. They also observed that activity of AKT, 4E-BP1, ERK2, S6, eEF2, and IF4B associated with S6K1 were regulated by insulin differently between fast- and slow growing chickens. Based on results from previous studies, mTOR pathway should play a critical role in insulin signaling and will have an effect on the phenotype of feed efficiency.

mTOR complexes

The target of rapamycin (TOR) was identified by mutations, TOR1 and TOR2, in yeast Scharomyces cerevisiae during a screen for resistance to rapamycin, which is related to immunosuppressive compounds (Heitman et al., 1991). TOR is known as FRAP, RAFT, RAPT, or mTOR in mammals and is an evolutionary conserved serine/threonine kinase complex. TOR coordinates cell growth and cell cycle progression through integrating both extracellular signals,

such as growth factors, and intracellular signals, such as nutrients (Fingar and Blenis, 2004; Shamji et al., 2003).

The characterization of TOR1 and TOR2 in yeast leads to the identification of two TOR protein complexes, rapamycin-sensitive TORC1 and rapamycin-insensitive TORC2. In mammals, mTORC1 and mTORC2 includes mTOR, raptor, and mLST8, and mTOR, rictor, and mLST8 respectively. mTORC1 regulates several pathway, such as protein synthesis, metabolism, ribosome biogenesis, transcription, and autophagy related to control of cell size (Wullschleger et al., 2006). In contrast, mTOR2 regulates the actin organization associated with determination of cell shape. It has also described that mTORC1 and possibly mTORC2 are controlled by growth factors (insulin/IGF), energy status of the cell, nutrients (amino acids), and stress (Wullschleger et al., 2006).

The downstream targets of mTOR

The ribosomal S6K1 and 4E-BP1 have been known well as the downstream targets of mTOR. They are controlled by changes in amino acid levels and glucose concentration (Hara et al., 1998; Inoki et al., 2003; Kim et al, 2002; Fingar and Blenis 2004). S6K1 is a member of the A, G, and C family of serine/threonine protein kinases (Hanks and Hunter, 1995). S6K1 is associated with nutrient pathways (e.g., amino acids and glucose) and insulin induces S6K1 activation by initiation by insulin receptor. It has also suggested that amino acid, especially leucine, dependent S6K1 activation is dependent on mTOR (Hara et al., 1998; Hay and Sonenberg, 2004; Kimball and Jefferson, 2004). With respect to energy metabolism, independent of alterations in amino acid levels, S6K1 is sensitive to changes in ATP levels via mTORC1 signaling (Dennis et al., 2001). S6 has been known as a downstream target of S6K1. On the other

hand, 4E-BP1 is one of eIF4E-binding proteins. elF4E (eukaryotic initiation factors 4E) is the cap-binding factor and by binding to the elF4E, 4E-BP1 is repressed in its non-phosphorylated state (Sarbassov et al., 2005; Richter et al., 2005). Phosphorylation of 4E-BP1 via mTORC1 signalling regulated by insulin releases eIF4E to restore cap-dependent translation (Richter et al., 2005; Proud 2006). In addition, eIF-4B and S6K1 induce dephosphorylation of the eukaryotic elongation factor 2 (EEF2). Thus, this pathway influences both the initiation and elongation stages of mRNA translation (Um et al., 2006, Dufner et al., 1999, Wang et al., 2001)

PI3K/Akt pathway

The phosphinositide 3'kinase(PI3K)/protein kinase B (PKB also called Akt), which encodes a serine/threonine kinase, mediates insulin signal pathway as a regulator of glucose transport, glycolysis, lipogenesis, glycogen synthesis, suppression of glucogenesis, cell survival, determination of cell size and cell-cycle progression (Whiteman et al., 2002). Akt is also a critical mediator of mTOR activity. In detail, by binding of insulin or insulin-like growth factors (IGFs) to their receptor, insulin receptor substrate (IRS) and PI3K are recruited (Wullschleger et al., 2006). Then, the binding of PI3K to IRS induces the conversion of phosphatidylinositol-4, 5-phosphate (PIP2) to PIP3 and by PIP3, PDK1 activates AKT (Wullschleger et al., 2006). In particular, Akt is regulated by mTORC2 and it has been reported that rictor of mTORC2 is essential for Akt activation in Dictyostelium (Sarbassov et al., 2005; Lee et al., 2005)

HIF/VEGF/mTOR pathway

Hypoxia-inducible transcription factor (HIF) is one of the pathways to be controlled by mTOR activity (Del Bufalo e al., 2006; Hudson et al., 2002). HIF, a heterodimeric transcription

factor, has been considered to play a role in adaptation to hypoxia and HIF-1α mRNA was increased by adipogenesis and insulin (He et al., 2011). HIF also affects angiogenesis in tumor development by increased expression of growth factors as transforming growth factor-(TGF), platelet-derived growth factor beta-(PDGF) and vascular endothelial growth factor (VEGF; Patel et al., 2006). Several studies demonstrated that HIF-VEGF-mTOR pathway exist in tumor development (Brugarolas et al., 2003; El-Hashemite et al., 2003a; Liu et al., 2003). The relationship between HIF1 or 2 alpha and mTOR complexes was also suggested (Toschi et al., 2008). HIF1 alpha is dependent on both mTORC1 and mTORC2, but HIF2 alpha is dependent only on mTORC2. Moreover, Mamane et al. (2004) proposed that signal of mTOR to VEGF can be dependent and independent on HIF.

mTOR pathway in chicken

Akt has been characterized in several tissues such as spinal cord motor neurons (Egea et al., 2001), granulose cells (Johnson et al., 2001) and muscles (Bigot et al. 2003a; Halevy et al. 2003). Bigot et al. (2003a) demonstrated that the phosphorylation of Akt on Ser473 is increased in neonatal early-feeding chicks. In the study, Akt phosphorylation was not found in 48h delayed-feeding chicks and only after food supply, Akt phosphorylation was increased. The role of mTOR in the activation of synthesis by amino acid in avian myogenic cell line (QM7) cells was also proposed (Tesseraud et al, 2003). Duchene et al. (2006) identified the activity of Akt/TOR/p70S6K pathway in chickens. In their result, refeeding and insulin treatment induced Akt, TOR, 4E-BP1, p70S6K and S6 in chicken muscle tissues. However, interestingly, the phosphorylation level of EEF2 was not changed by refeeding and insulin conditions.

S6K1 (known as P70S6 kinase) has been identified in quail myogenic cell line (QM7) and in chicken muscles (Tesseraud et al., 2003; Bigot et al., 2003a and b). Interestingly, P70S6K phosphorylation and protein systhesis were dependent on the availability of individual amino acids (methionine and leucine; Tesseraud et al., 2003). Furthermore, P70S6K activity in chicken muscles was increased after re-feeding for 30 min following 16h starvation and after one insulin treatment (Bigot et al., 2003b) and P70S6k activation was dependent on insulin in post-hatched chick muscles (Bigot et al., 2003a). It has been also reported that the activity pattern of S6K1 regulated by insulin was different between fast- and slow-growing chicken muscles (Duchene et al., 2008). In muscles, S6K1 activation was more sensitive for insulin treatment in the fast-growing group than in the low-growing group. However, in liver tissues, the S6K1 activation was not dependent on insulin treatment or genotype such as fast- and slow growing group. In the study, although they observed the difference of AKT, 4E-BP1, ERK2, S6, eEF2, and IF4B activity induced by insulin between fast- and low growing chickens, the activity of the genes in both of muscle and liver was not modified between two lines.

Nitrogen recycling metabolism

Animals take in nutrients and excrete waste products. Among nutrients, amino acids are different from carbohydrates and lipids because excess amino acids (e.g. excess of the amount required for growth and maintenance) cannot be stored in animal. However, the excess amino acid can be degraded and excreted as nitrogenous waste. In cells, continuous synthesis and degradation occurs. It determines protein balance in tissues and is termed protein turnover. Previously, a possible connection between amino acids and protein turnover has been suggested. Various catabolic conditions (e.g., injury, sepsis, etc) indicating negative protein balance in

skeletal muscle decreased glutamine level (Curthoys and Watford 1995; Clowes et al. 2005). Maclennan et al. (1987) and (1988) showed that by infusion of glutamine in rat muscle, protein synthesis was increased and protein breakdown was inhibited.

The metabolic processes of protein turnover require an input of energy (e.g. ATP). Because both synthesis and breakdown need energy, an increase in protein mass concurs with a reduction in rates of protein breakdown, rather than by simply increasing rates of protein synthesis or turnover (Muramatsu and Okamura 1985; Muramatsu et al., 1987). In addition, reduced rates of protein degradation allow an increase in lean body mass without a proportionate increase in maintenance energy needs. In broilers and rats, feed efficiency in selected lines was inversely correlated with rates of muscle protein breakdown (Tomas et al., 1988 and 1991; Tomas and Pym 1995). Therefore, these results could suggest that feed efficiency phenotype can be influenced by protein turnover, which is linked to amino acid metabolism.

Ammonia metabolism

Amino acids are usually catabolized in liver and converted into nitrogen and intermediates for the TCA cycle. When glucose level is low, amino acids, especially alanine and glutamine are supplied from muscle to liver. The first step in amino acid gluconeogenesis for most L-amino acids is their transamination to form glutamate, catalysed by a group of transaminase enzymes in the cytosol of hepatocytes. The cytosolic glutamate functions as a major pool of liable nitrogen. In mammals, two transport systems for glutamate in the inner membrane are known: 1) an H^+ symporter, and 2) an aspartate-glutamate antiporter (LaNoue, N.F. and Schoolwerth, 1979; Njogu and Hoek, 1983). The H^+ symporter deaminates glutamate via glutamate dehydrogenase (GDH), located in the mitochondrial matrix, forming NH_4^+ and α-

ketoglutarate (McGivan and Chappell, 1975). Human expresses two isoenzymes of GDH, glutamate dehydrogenase 1 (GLUD1) and GLUD2 (Mastorodemos et al., 2009). In the second transport system for glutamate, aspartate-glutamate antiporter transaminates glutamate with oxaloacetate forming aspartate via the mitochondrial isozyme of aspartate aminotransferase (Vorhaben and Campbell, 1977). Two isoenzymes of the aspartate aminotransferase are glutamic-oxaloacetic transminase (GOT1), a cytosolic isoenzyme, which is derived mainly from red blood cells and heart, and GOT2, the mitochondrial isoenzyme, which presents predominantly in liver.

Another major route for nitrogen metabolism is the conversion of glutamate into glutamine by glutamine synthetase (GS; Newsholme et al., 2003). It is the primary ammonia-detoxifying process by GS and NH_3 is the substrate for GS [glutamate ->ammonia -> glutamine (amid)]. The reverse reaction is also catalyzed by glutaminase (GLS; Curthoys et al., 1995). In chicken liver, GS is expressed in all hepatocytes (Smith et al., 1987 and 1988).

There are aminotransferases for all amino acids except threonine and lysine. In most of transamination reactions, glutamate and α-ketoglutarate commonly play a role with many different aminotransferases. Serum aminotransferases such as aspartate aminotransferase, AST (serum glutamate-oxaloacetate-aminotransferase, SGOT) and alanine transaminase, ALT (also called serum glutamate-pyruvate aminotransferase; SGPT) have been known as clinical markers of tissue damage. Alanine transaminase participates in the transfer of skeletal muscle carbon and nitrogen (e.g., alanine) to the liver. In the glucose-alanine cycle, pyruvate is transaminated to alanine in muscle and the ammonia is transferred to α-ketoglutarate by alanine transaminase in liver and finally regenerates pyruvate. Another useful amino acid-related reaction is the amidation of aspartic acid and it produces asparagines by asparagine synthase.

Uric acid metabolism

Uric acid is produced based on the metabolism of adenine- and guanine- based purine. Especially, in bird, uric acid is the end product of nitrogen excretion. First, purine ring is synthesized from a ribose-phosphate backbone donated by 5-phophoribosyl 1-pyrophosphate (PRPP) by PRPP synthetase. It is catalyzed by the enzyme, amido phosphoribosyltransferase with glutamine. Another pathway of purine nucleotide synthesis occurs by two enzymes, adenine phosphoribosyltransferase and hypoxanthine-guanine phosphoribosyltransferase (HGPRT) catalyzing reactions between PRPP and the respective purine base substrates. In the final step, purine is catalyzed into hypoxanthine and xanthine sequentially by xanthine dehydrogenase and as the result, uric acid is generated. In bird, guanine is the major excretory product (Anderson, 1965)

Environmental benefits of improving feed efficiency.

The agricultural sector contributes to the sources of greenhouse emission worldwide. In USA, Canada, and Australia, livestock takes part 40, 50, and 68% in their national agricultural green-house gas emission (AGO, 2004; Environment Canada, 2004; US-EPA, 2005). Animals produce greenhouse gases, methane and nitrous oxide. The greenhouse gases can be global warming potential 21 and 310 times greater than carbon dioxide, respectively (AGO, 2001). It has been reported that high efficiency (low RFI) steers showed 21 and 15% lower methane emission and manure production than for low efficiency (high RFI) steers (Okine, 2001). A study by Herd et al. (2002) estimated that methane and nitrous oxide production from fermentation of feces was 15 and 17% lower in low RFI cattle. Also, cattle with high RFI produced 15% more methane per day than those selected for low RFI. However, those studies used estimated (not

measured) methane emission values. Nkrumah et al. (2006) measured actual methane production. It was shown that methane production was 28 and 24% less in low-RFI animals than in high- or medium -RFI animals, respectively.

In addition, it is known that nitrogen (N) and phosphorus (P) in animal manure are the most harmful components. This means that improving nutrient efficiency of animals would invariably decrease excretion of these environmental contaminations. Although it is not clear that feed efficiency is correlated to nitrogen retention (i.e, nitrogen amount in animal manure), animals with high feed efficiency are expected to covert more energy and nitrogen into body protein (Zhanga and Aggrey, 2003). If the correlation between feed efficiency and nitrogen retention is demonstrated clearly in the future, low nitrogen in animal manure by improved feed efficiency would produce environmental benefits.

Table 2.1 Definitions for feed efficiency

Trait name	Abbreviation	Formula
Feed conversion ration	FCR	Feed intake/weight gain per day
Maintenance efficiency	ME	Body weight/ feed intake at zero body weight change
Partial efficiency of growth	PEG	Weight gain per day/(feed intake –maintenance feed requirements)
Residual feed intake	RFI	Feed intake-expected feed requirements, where the expected feed requirements was obtained by the regression of feed intake on average test period liveweight 0.75 and weight gain per day

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CHAPTER 3

Transcriptomic analysis to elucidate the molecular basis of feed efficiency in meat-type chickens[1]

[1]Lee, J. and S. Aggrey (2012) To be submitted to the journal related to genetics.

ABSTRACT

The feed efficiency phenotypes defined by genotypes or gene markers are unknown. To date, there are only limited studies on global gene expression profiling on feed efficiency. The objective of this study was to identify genes and the pathways associated with residual feed intake (RFI) through transcriptional profiling of duodenum at two different ages in a chicken population divergently selected for low (LRFI) or high (HRFI) RFI. The global gene expression differences in LRFI and HRFI were assessed by the Affymetrix GeneChip® Chicken Genome Array and RT-PCR using duodenal tissue at days 35 and 42. The Ingenuity Pathway Analysis program was used to identify canonical and gene network pathways associated with RFI. A global view of gene expression differences between LRFI and HRFI suggests that RFI can be explained by differences in (1) cell division, growth, proliferation and apoptosis, (2) protein synthesis, (3) lipid metabolism, and (4) molecular transport of cellular molecules. Chickens selected for improved RFI achieve efficiency by reducing feed intake with a nominal or no change in weight gain up-regulating CD36, PPARα, HMGCS2, GCG and down-regulating PCSK2, CALB1, SAT1 and SGK1. Chickens selected for reduced RFI via reduced feed intake with no change in weight gain achieve feed efficiency for growth by up-regulation of genes that reduces appetite with increased cellular oxidative stress, prolonged cell cycle, DNA damage and apoptosis in addition to increased oxidation of dietary fat and efficient fatty acids transport from the intestines.

Key Words: Residual feed intake, microarray, RT-PCR, gene network, chickens,

INTRODUCTION

Feed constitutes a major portion of total production costs, therefore, improvement in the efficiency of feed utilization will reduce the amount of feed required for growth, which would directly reduce production cost, increase profitability, and subsequently reduce the amount of manure produced. Genetic variation in feed efficiency still exists in broiler chickens and needs to be exploited for genetic gain (Zhang and Aggrey, 2003). However, current improvement methods for feed efficiency (FE) are limited in the rate of annual genetic improvement because the actual feed efficiency phenotypes (FEP) defined by genotypes or gene markers are unknown. This is because the gene network mechanisms that underlie FE are unknown. In addition, FE is a compound trait comprising of feed intake (FI) and tissue accretion traits, and gene that affect either FI or body weight gain (BWG) may or may not necessarily affect FE. Thus, selection programs based on combinations of feed efficiency genotypes (FEG) or gene markers and the current traditional method will offer greater accuracy in breeding value estimation and consequently, a faster rate of genetic improvement.

Some researchers have used quantitative trait loci (QTL) mapping to show complex genetic basis for feed efficiency. Feed efficiency QTLs have been mapped in beef cattle (Sherman et al., 2009; Márquez et al., 2009), pigs (Rothschild et al., 2007) and in poultry (van Kaam et al., 1999; De Koning et al., 2004). However, confident interval of QTL regions are usually large and further fine mapping is required to narrow the QTL region and subsequently identify the underlying genes. Genomic profiling is the first critical step to comprehensive understanding of the mechanisms that underlie the interaction of nutrition and the genome. It is well known that nutritional perturbations affect gene expression (Cogburn et al., 2004; Maloney et al., 2005; Huang et al., 2007), and these perturbations have been used to establish gene

networks. However, gene networks established from genetic mutation perturbations would be useful for genetic improvement since such functional mutations are the genetic raw material needed to establish trait genotypes. There has been very scant information on specific genes or gene networks that underlie feed efficiency. To date, there has been only a limited study on global gene expression profiling on feed efficiency (Kong et al., 2011). Microarray technology permits genome-wide differential gene expression analysis to uncover pathways and networks underlying feed efficiency.

The objective of this study was to identify genes and the pathways associated with residual feed intake (RFI) through transcriptional profiling of duodenum at two different ages in a chicken population divergently selected for low (LRFI) or high (HRFI) RFI. Aggrey et al. (2010) have shown that RFI at day 28-35 is genetically correlated to BWG whereas RFI at day 35-42 is not correlated to BWG. These two ages would potentially delineate the underlying mechanisms of FE at different stages of growth in the meat-type chicken.

MATERIALS AND METHODS

Experimental Population and animal husbandry

The chicken population were bred and raised at the Poultry Research Center of the University of Georgia under permit from the animal care and use committee of the University of Georgia. From the Arkansas random bred population the low (LRFI) and high (HRFI) RFI lines were divergently selected for RFI at day 35-42 based on their breeding values for 3 generations. The differences in the divergent lines are shown in Table 3.1. All birds were raised under conditions described by Aggrey et al. (2010). Duodenum tissues were collected from 16 male

chickens under sterile conditions and immediate frozen in liquid nitrogen. Tissues were collected from 4 males at days 35 and 42 in each line.

RNA preparation and microarray experiments

Total RNA was extracted from the duodenum tissues using Trizol reagents (In Vitrogen, Carlsbad, CA) and purified with RNeasy mini kits (Qiagen, Valencia, CA) according to the manufacturer's protocol. The RNA samples were suspended in RNase-free water and sample purity and concentration were measured on an Agilent Bioanalyzer (Agilent Technology, Santa Clara, CA) and stored at -86^{o}C until used. Five micrograms of total RNA from each individual was used for the gene expression analysis using the Affymetrix GeneChip® Chicken Genome Array (Affymetrix, Santa Clara, CA) according to manufacturer's protocol. The Affymetrix Array contains a comprehensive coverage of 32,773 transcripts corresponding to 28,000 chicken genes and controls. The chicken Genome Array also contains 689 probe sets for detecting 684 transcripts from 17 avian viruses). The control sequences include: Hybridization controls (*bioB, bioC, bioD, nad cre*), Poly-A controls (*dap, lys, phe, and thr*), and Housekeeping control genes (eukaryotic translation elongation factor 1 alpha (EEF1A1), beta Actin and GAPDH).

Analysis of microarray data:

All array data was saved as raw image files and converted into probe data using algorithm MAS5 in the Affymetrix software (Liu et al., 2002). The probe set data from all arrays were simultaneously normalized using quantile normalization, which eliminates systematic differences between arrays without significantly altering the relative intensity of probes within an array (neutralizes the effects of background noise and the processing artifacts). Then, the mean

optical background level for each array was estimated, and the intensity for each probe was adjusted to remove this. The program uses each probe's sequence information to adjust for measured intensity for the effects of non-specific binding due to the differences in bond strength between two types of base pairs. Hereafter, the normalized, background-corrected data was transformed to the $\log_2$ scale and the median polish procedure was used to combine multiple probes into a single measure of expression for each gene on each array. The output of this step was analyzed by mixed model. The following mixed linear model was used to analyze the probe level intensities for every gene separately: $y_{ijk} = \mu + P_i + L_j + Ag_k + (L * Ag)_{jk} + A_l + e_{ijkl}$; where y_{ijkl} is the $\log_2$ transformed intensity for probe P_i $(i = 1,2,...20)$ generated under line L_j ($j = 1 = LRFI, 2 = HRFI$)) and age group Ag_k $(k = 1,2)$ in array A_l $(k = 1,2,...,16)$ and e_{ijkl} is the residual term. We assumed that the distribution of the random effects in the model: $e_{ijkl} \sim N(0, \sigma_e^2)$ and $A_l \sim N(0, \sigma_a^2)$; where σ_e^2 and σ_a^2 are the within and between array variances, respectively. Both variance components are unknown and maximum likelihood (ML) based methods were used for their estimation. The ML residual estimates were combined with the least-square mean difference of the two RFI lines to provide t-test statistics on within-age and between age-groups for each gene. The resulting *p*- values that resulted from these tests for all genes were corrected for multiple testing for false discovery rate of 5% (Hochberg and Benjamini, 1990).

Real-time reverse-transcriptase polymerase chain reaction assay

Two micrograms of total RNA was reversed transcribed with high capacity cDNA reverse transcription kits according to manufacturer's protocol (Applied Biosystems, Carlsbad, CA, USA). Real-time PCR reactions were performed using the StepOnePlus (Applied

Biosysems, Carlsbad, CA). 0.5µl of cDNA served as a template in a 20µl PCR mixture containing 0.3µl each of forward and reverse primers from 10 µM stocks and 2X Fast SYBR Green Master Mix (Applied Biosystems, Carlsbad, CA). The PCR conditions were 95°C for 20 seconds, followed by 40 cycles of 95°C for 3 seconds and 60°C for 30 seconds. In addition, at the end of each reaction, a melting temperature curve of every PCR reaction was determined. PCR runs for each of the 16 samples were performed in duplicates. Data were analyzed according to $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001) and were normalized by β-actin expression in each sample. Differential mRNA expression was expressed as LRFI/HRFI. The NCBI accession numbers, forward and reverse primers of genes used in this study are provided in Table 3.2

Functional and pathway analysis of differentially expressed genes

The molecular, cellular and biological functional interpretations of differentially expressed genes between lines and within ages were investigated using Gene Ontology (GO, http://www.-geneontology.org/). Differentially expressed genes (FDR $P \leq 0.05$) and fold change of 1.2 were considered significant. The Ingenuity Pathway Analysis program (Ingenuity Systems Inc., Redwood City, CA) was used to identify canonical and gene network pathways associated with the differentially expressed genes.

RESULTS

The descriptive statistics of the growth and feed related traits for LRFI and HRFI are presented in Table 3.1. At day 28-35, body weight gain (BWG) and feed intake (FI) and RFI were significantly lower in LRFI compared to HRFI, however, at day 35-42, metabolic body

weight (MBW), FI and RFI were significantly lower in LRFI compared to HRFI, and there was no difference in BWG. There were 158 genes differentially expressed between LRFI and HRFI regardless of age with gene expression fold change ranging from -2.8 to 3.4. About 35% of the genes were down-regulated and 65% up-regulated in LRFI. There were 418 genes that were differentially expressed only at day 35 and also 274 genes that were differentially expressed at day 42. In general, there were significantly higher amount of genes that were up-regulated than down-regulated in LRFI compared to HRFI.

Genes that were significant with at least 1.2 fold change were subject to annotation and pathway analyses using the Ingenuity software package (IPA, www.ingenuity.com). From GO, the main biological functions that distinguish LFRI from HRFI regardless of age are: lipid metabolism, small molecule biochemistry, molecular transport, cell death and protein synthesis (Table 3.3).

The mRNA levels of 10 genes from the categories of the biological functions were subject to RT-PCR for confirmation using the same individuals used in the microarray analysis. Comparing LRFI with HRFI we confirmed the up-regulation of ALB, BBOX1, FGF19 and PPARA gene and down-regulation of KCNJ15, LYPLAL1, SAT1 and STEAP4 (Table 3.3). Each of the 5 main biological functions associated with LRFI/HRFI in Table 3.2 was overlaid onto the global molecular network developed from information from the databases of the Ingenuity Pathway Knowledge Base. The gene network representing the molecular relationships between genes or gene products for amino acid metabolism and molecular transport is presented in Figure 3.1.

DISCUSSION

Unlike many complex traits govern by several interacting genes, feed efficiency is rather a compound trait comprising of an interaction between feed- and growth- related traits. This also means that, the genetic architecture of FEP may be different among populations. From the genetic parameters of the base population the divergent lines were derived (Aggrey et al., 2010), and results from LRFI and HRFI lines (Table 3.1) show that the characteristics of RFI at day 28-35 is different from that of day 35-42. Selecting on RFI at day 35-42 in the current population improved feed efficiency at day 28-35 by reducing FI and to a smaller extent BWG, and also improved feed efficiency at day 35-42 by reducing FI without changes to BWG. The differences in the genetic relationship between RFI parameters between the two periods could explain the differences in the gene expression patterns. We would focus more on the gene expression differences between the divergent lines than the age specific differences.

A global view of gene expression differences between LRFI and HRFI suggests that RFI can be explained by differences in (1) cell division, growth, proliferation and apoptosis, (2) protein synthesis, (3) lipid metabolism, and (4) molecular transport of cellular molecules. Unlike pigs where RFI can be corrected for back-fat prior to selection, correction for abdominal fat (ABF) cannot be made in chickens because appropriate and reliable in-vitro measurement methodology is lacking. The HRFI line has a slightly higher ABF than the LRFI line pointing differences in lipid metabolism between LRFI and LRFI (Table 3.1).

Lipid metabolism and transport

In the duodenum of LRFI chicken there was a higher expression of thrombospondin receptor (CD36), Peroxisome proliferator-activated receptor-alpha (PPARα), albumin (ALB),

fibroblast growth factor 19 (FGF19), nuclear receptor (NROB2), microsomal triglyceride transfer protein (MTTP), choline kinase alpha (CHKA), and alipoprotein H (APOH) which are involved in lipid, cholesterol and bile metabolism, and transport. CD36 is a plasma membrane glycoprotein expressed by a broad variety of tissues including intestines (Harmon and Abumrad, 1993). CD36 is receptor-like protein that binds to oxidized LDL and thrombospondin (Silverstein et al., 1989). It also displays a very high affinity for long-chain fatty acids (Abumrad et al., 1993). Feeding can stimulate intestinal mucosa cells to produce the lipid messenger fatty-acid ethanolamide, oleoylethanolamide (OEA), which when infused as a drug reduces FI by engaging PPARα (Schwartz et al., 2008). OEA is an endogenous agonist of PPARα (Fu et al., 2005). In mice lipid binding to duodenal CD36 is sufficient to produce satiety effect and thereby modulating FI (Naville, et al., 2012) whereas infusion of carbohydrate or protein does not. Activation of intestinal OEA mobilization, enabled by CD36 mediates uptake of dietary fat and serves as a molecular sensor linking fat ingestion to satiety, and targeted disruption of CD36 or PPARα abrogates the satiety response to dietary fat (Schwartz et al., 2008). The up-regulation of PPARα is also consistent with the report of Madsen and Wong (2011) who demonstrated that this gene is expressed in intestines of chickens subjected to restricted feeding.

The birds used in the current study were fed *ad libitum*. It is possible that the reduced FI in LRFI compared to HRFI was due to up-regulation of CD36, PPARα and other genes are involved appetite suppression. PPARα also regulates the expression of genes involved in FA oxidation and has triglyceride (TG) lowering and HDL cholesterol elevating effects (van Raalte et al., 2004) .The hepatic leukemia factor (HLF) was up-regulated in LRFI. HLF encodes a subset of bZIP transcription factors (Inaba et al., 1992). Gachon et al. (2011) showed that HLF modulates the activity of PPARα by contributing to cyclic release of FAs from thioesters which

act as ligands for PPARα. The up-regulation of 3-hydroxy-3-methylglutaryl-CoA synthase 2 (mitochondrial) (HMGCS2) in LRFI suggests that energy derived from carbohydrates is inadequate resulting in increased lipid-derived energy (Nadal et al., 2002). Another negative regulator of appetite, glucagon (GCG) which is involved in the regulation of carbohydrate, insulin secretion, lipid metabolism, and in the secretion of gut endocrine cells that promote nutrient absorption (Cummings and Overduin, 2007; Lemmens et al., 2011) was highly expressed in LRFI. . It appears that there is increased lipid oxidation in LRFI compared to HRFI with up-regulation of lipid transporters such as ALB, APOH, MTTP, and CHKA suggesting the need to scavenge available luminal FA from the intestines. This may reflect the low abdominal fatness in LRFI compared to HRFI. On the hand, aldose keto reductase 1D1 (AKR1D1) essential for bile acid biosynthesis, and colipase (CLPS), a pancreatic lipase for efficient dietary lipid hydrolysis were both up-regulated in HRFI.

Protein synthesis, Cell growth and Apoptosis

Chickens selected for improved RFI achieve efficiency by reducing FI significantly with a nominal or no change in BWG suggesting that the dynamics of growth (protein metabolism, cell division, proliferation and apoptosis) would be different between LRFI and HRFI. The dynamics of interacting gene network of genes differentially expressed and of those with no expression differences are depicted in Figure 3.1. Ataxin 3 (ATXN3), phenylalany-tRNA synthetase (FARSB), metallothionein 3 (MT3), myeloid cell leukemia sequence 1 (MCL1), CHKA, FGF19, serpine peptidase inhibitor (SERPINE2), syntaxin 12 (STX12), lectin, galactoside-binding soluble 8 (LGALS8), mitogen-activated protein kinase kinase 4 (MAP2K4), and carboxypeptidase 2(CPA2) were up-regulated in LRFI whereas proprotein convertase

subtilisin/kexin type 2 (PCSK2), calbindin 1 (CALB1), deoxyribonuclease 1 (DNASE1), maternal embryonic leucine zipper kinase (MELK), spermidine/spermine-N (1) acetyltransferase 1 (SAT1), serum/glucocorticoid regulated kinase 1 (SGK1) were up-regulated in HRFI. It appears that the chickens in the LRFI line were maintaining their growth through protein homeostasis maintenance and apoptosis. The up-regulated gene ATX3 is a deubiquitinating enzyme involved in protein homeostasis maintenance, transcription, cytoskeleton regulation, and interacts with key regulators of transcription and represses transcription and acts as a histone-binding protein that regulates transcription (Li et al., 2002) while CHKA suppresses PI3-kinase/ATK signaling and disrupt actin cytoskeleton organization and serves as a sensor to assess DNA damage in the cell cycle (Ramirez de Molina et al., 2008). DNA damage could be high in the intestinal cells of LRFI leading to elimination of target cells through apoptosis. The OSGIN1 which is up-regulated in LRFI is a growth factor that encodes an oxidative stress response protein that regulates apoptosis and expression of OSGIN1 is regulated by p53, and is induced by DNA damage (Wang et al., 2005).

Kolath et al. (2006) reported higher oxidative stress in the muscle tissue of LRFI compared to HRFI Angus steers. However, Bottje's group (Bottje et al., 2002; Iqbal et al., 2004, Ojano-Dirain et al., 2007a; Kong et al., 2011) has alluded that increased oxidative stress and protein oxidation due to increased mitochondrial reactive oxygen species (ROS) is associated with low-FE phenotype. It should be pointed out that the definition of FE by Bottje's group was BWG per FI and FE was explained by changes in BWG and not FI (Iqbal et al., 2005). Low-FE is the same as high FCR and even though FE/FCR and RFI are used in the literature to describe feed efficiency, they reflect slightly different biological phenomenon (Aggrey et al., 2010). The MAP2K4 gene was up-regulated in LRFI encodes a member of the MAP kinase signaling family

that is involved in gene expression, cell proliferation and apoptosis (Chang and Karin, 2001). When activated MAP2K4 can phosphorylate JNK or p38 with dual specificity, resulting in the activation of the stress activated protein kinase (SAPK) pathway, which has been associated with apoptosis and neoplastic transformation (Davis, 2000). Also, LGALS8 which was up-regulated in LRFI has been shown binds to integrin to inhibit cell adhesion cell survival and induces apoptosis (Hadari et al., 2000).

The myeloid cell leukemia 1 (MCL1), an anti-apoptotic member of the B-cell lymphoma 2 (BCL2) family of proteins (Millman and Pagano, 2011) was up-regulated in LRFI. However, Youle and Strasser (2008) has shown that during prolonged mitotic arrest, MCL1 is degraded to induce apoptosis, and interestingly, the ubiquitin-proteasome system (UPS18) which plays a key role in dictating the intrinsic apoptotic pathway in cells arrested in mitosis was also up-regulated in LRFI. Also CALB1 was down-regulated in the duodenum of LRFI suggesting a putative slow-down of Ca2+ transport out of the duodenum. Nemere et al. (1991) demonstrated that inhibition of calcium absorption through down-regulation of proteins involved in the transcellular pathway, as a consequence of oxidative stress triggers mitochondria mediated apoptosis. Since the LRFI is achieving efficiency in feed utilization through reduced FI and not BWG, it is reasonable to argue that birds in the LRFI maintain their weight gain via disruption and prolongation in the cell cycle resulting in DNA damage which triggers oxidative stress and consequently apoptosis.

The DNASE1, MELK, PCSK2 and SAT1 genes were up-regulated in HRFI. PCSK2 is a serine protease responsible for endoproteolysis of secretory precursor proteins including proinsulin, proopiomelanocortin, growth factors, cytokines and transcription factors (Seidah and Chretien, 1999). PCSK2 action points to increased cell proliferation and growth in HRFI. Recently, Gagnon et al. (2011) reported that PCSK2 is also involved in satiety-regulating

peptides and intestinal motility. ATP-binding cassette, sub-family B (MDR/TAP), member 1 (ABCB1) gene was up-regulated in HRFI. The ATP-binding cassette (ABC) family of proteins comprises a group of membrane transporters involved in the transport of a wide variety of compounds, such as xenobiotics, vitamins, lipids, amino acids, and carbohydrates in the gut (Mutch et al., 2004). Potassium ions are required for gastric acid secretion. Several potassium channels have been implicated in providing K(+) at the apical membrane of parietal cells. In examining the mRNA expression levels between gastric mucosa and liver tissue in mice, KCNJ15 stood out as the most highly specific K(+) channel in the gastric mucosa (He et al., 2011). KCNJ15 was up-regulated in HRFI and has been found to regulate gastric acid production (Kaufhold et al., 2008). Gastric acid is required to create the acidic environment required for protein digestion. It is possible that, there is increased protein hydrolysis coupled with increased nutrient transport across the duodenum in HRFI. DNASE1 is an extracellular endonuclease secreted by digestive glands and is actively involved in DNA fragmentation and/or apoptosis (Eulitz and Manherz, 2007). SAT1 which regulates cellular polyamine content (Pegg, 2008) is up-regulated in HRFI. Polyamines are involved in cell growth and differentiation (Casero and Pegg, 1993). Pegg (2008) noted that, other effects of increased SAT1 activity include death of pancreatic cells, blockage of regeneration of tissue growth. MELK (maternal embryonic leucine zipper kinase), a cell cycle dependent protein kinase was up-regulated in HRFI. It has been known that MELK is involved in diverse cell processes including cell proliferation, apoptosis, cell cycle and mRNA processing (Badouel, et al., 2010). How HRFI maintain their body weight is less clear, but it appears that, a combination of cellular differentiation, cell growth, apoptosis and lack of tissue generation culminate to maintain weight gain despite increased FI.

Cellular Assembly and Organization, and cell-to cell signaling

At day 28-35 cellular assembly and organization, and also cell to cell signaling interactions were different between LRFI and HRFI. Chemokine receptor 4 (CXCR4), islet amyloid polypeptide (IAPP) and insulin (INS) were down-regulated whereas aurora A kinase (AURKA), chemokine ligand 12 (CXCL12), heat shock 70KDa protein 5 (HSPA5), purinergic receptor P2X (P2RX1), telomeric repeat-binding factor 1 (TERF1), mitogen-activated protein kinase kinase 7 (MAP3K7), tumor necrosis factor receptor superfamily, member 6B (TNFRSF6B), tumor necrosis factor superfamily family, member 10 (TNFSF10), target protein for Xklp2 (TPX2), vascular enthothelial growth factor A (VEGFA) were up-regulated in LRFI. Taken together, most of these genes control a variety of cell functions, including mitosis, transcription, genome stability, transcription regulation, cell proliferation, cell migration and apoptosis. Coincidentally, some of the genes differentially expressed are also involved in various forms of cancers either inducing or inhibiting cell proliferation and apoptosis (Kuribayashi et al., 2008; Jiang et al., 2010; Chang, et al., 2012). Differential cell growth and apoptosis between LHRFI and HRFI may have contributed to the weight gain differences at day 28-42.

Vitamin Metabolism

At day 35-42 there was a difference in vitamin metabolism function between LRFI and HRFI. Acetoacetyl-CoA synthetase (AACS), acetyl-coA acetyl transferase 2 (ACAT2), APOB, insulin induced gene 1 (INSIG1), retinol dehydrogenase 12 (RDH12), squalene epoxidase (SQLE) were down-regulated while acyl-CoA oxidase 2 (ACOX2), angiotensin (AGT), group-specific component (Gc), retinol binding protein 4 (RBP4), microsomal triglyceride transfer protein (MTTP), steroid sulfatase (STS) and transthyretin (TTR) were up-regulated in LRFI.

Most of these genes are involved with cellular cholesterol levels, biosynthesis of cholesterol esters, fatty acids, sterols, and also vitamins A binding protein and vitamin D transport. Vitamins are required in maintaining many essential physiological processes and may also serve as components of coenzyme systems. Differential expression in the function of vitamin metabolism most likely pertains to the fat metabolism since weight gain was similar, and abdominal fatness was different between LRFI and HRFI.

CONCLUSION

Feed efficiency can be achieved by combinations of (1) reduced FI with no change in weight gain, (2) increased weight gain with no change in FI, or (3) reduced FI with increase in weight gain, all of which reflect different molecular and physiological underpinnings. Researchers on feed efficiency should be strict and consistent with their definitions, and also age of animals and time span of the study in order to ascertain the paradigm of feed efficiency that they are elucidating. The whole complexity of genes involved with RFI may never be known, but from a simplistic view, it appears that chickens selected for reduced RFI via reduced FI with no change in weight gain achieve feed efficiency for growth by up-regulation of genes that reduces appetite with increased cellular oxidative stress, prolonged cell cycle, DNA damage and apoptosis in addition to increased oxidation of dietary fat and efficient FA transport from the intestines. Genetic markers could be developed in these genes to assist in the improvement of RFI.

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Table 3.1 Means (±SE) of feed efficiency parameters in a chicken population divergently selected for low (LRFI) or high (HRFI) residual feed intake

Trait	Period	LRFI	HRFI	Pr>F
MBW	28	171.43±1.15	173.99± 1.27	0.1357
BWG	28-35	394.37±4.90	420.89± 5.42	0.0003
FI	28-35	707.89±8.65	803.15± 9.58	<0.0001
RFI	28-35	-31.86±6.38	32.33± 7.07	<0.0001
FCR	28-35	1.80±0.02	1.93± 0.02	<0.0001
MBW	35	222.20±1.47	227.81± 1.62	0.0103
BWG	35-42	376.39±4.72	372.09± 5.23	0.5406
FI	35-42	776.60±9.96	886.72±10.91	<0.0001
RFI	35-42	-41.35±8.33	54.80± 9.22	<0.0001
FCR	35-42	2.13±0.04	2.49± 0.05	<0.0001
ABFW	42	28.93±0.64	30.52±0.69	0.0943
ABFY (%)	42	2.60±0.08	2.81±0.09	0.0885

[1]MBW = Metabolic body weight (BW); BWG = BW gain; FI = feed intake; FCR = feed conversion ratio; RFI = residual feed intake; ABFW=abdominal fat weight; ABFY=abdominal fat yield

[2]All traits measured in (g) except for FCR which was (g/g);

[3]Age (d) or age range (d) that trait was measured

Table 3.2 Primer pairs used in quantitative RT-PCR analysis and size of product

[1]Gene Symbol	GenBank Accession No.	Forward Primer	Reverse Primer	Size
BBOX1	XM_424543.2	AGT GGC TGT GCA CTC TGA AGA AGA	AGT GAG ACG CAG GAA ACC AAT CCT	110bp
FGF19	NM_204674.1	AGT GGA GGT GAC AGA CTT TGG TGA	CCG CGT GTG CAA TCA GAG TCA TTT	169bp
ALB	NM_205261.1	TAC TAA GTG CTG CCA GCT TGG TGA	AGC TGA CTG CAG CAT TGT GAA ACG	138bp
LYPLAL1	XM_419411.3	TGT GCC AAG GGC TTA CAG ACT TGA	AAA TGC ATT GCC ATT CCA CCT CCC	109bp
STEAP4	XM_001235256.2	ATG GCA TCT GCT CCT CTT CAC TGT	AGA GCT TTC CCT CGG TTA ATG CCT	149bp
PCSK2	XM_419332.3	AAC AAC CAC TAC AGG TTC CCA GGT	AGT GCT TCA GTA GGT CTG CTG CTT	111bp
SAT1	NM_204186.1	TGA AGA AAC AGC TGC TCC TCC TGT	TGG CTA GTT CCT TGA TCA GTC GCA	155bp
KCNJ15	XM_425554.3	AGT CCA CCA GTG CTG TCT GTC AAA	AGA GAG AGA AAC CAC AGG CAC GAA	92bp
TMEM135	XM_417208.3	AGG CAG CTG TCA TGG AAG TTC AGA	TCT TGG GTC AAG CTT CGG AGT GAA	164bp
PPARA	NM_001001464.1	TGG GAT GCT GGT AGC CTA TGG AAA	ACC AGG ACG ATC TCC ACA GCA AAT	187bp
β-actin	NM_205518.1	AGA CAT CAG GGT GTG ATG GTT GGT	TCC CAG TTG GTG ACA ATA CCG TGT	125bp

[1]The genes are used for real-time RT-PCR analysis are: butyrobetaine (gamma), 2-oxoglutarate dioxygenase (BBOX1); fibroblast growth factor 19 (FGF19), albumin (ALB); lysophopholipase-like 1 (LYPLAL1); STEAP family member 4 (STEAP4); protein convertase subtilisin/kexin type 2 (PCSK2); spermindine/spermine N1-acetyltransferase 1 (SAT1); potassium inwardly-rectifying channel, subfamily J, member 15 (KCNJ15); transmembrane protein 135 (TMEM135); peroxisome proliferator activated receptor alpha (PPARA); and beta actin (β-actin).

Table 3.3 Molecular and cellular functions between a chicken population divergently selected for low (LRFI) or high (HRFI) residual feed intake

Category	P-value	Genes[1]
Lipid Metabolism	9.38E-06-3.23E-02	**AADAC**, ABCB1, **ALB**, **ALDH8A1**, **AP2M1**, **APOH**, AKR1D1, ATP11B, CALB1, **CD36**, **CHKA**, CLPS, **CYP1A1**, CYP2C18, **CYP2C9**, **DLD**, **EPHX2**, **FGF19**, **GCG**, **GstA4**, **HLF**, **HMGCS2**, **IL8**, **LGALS8**, **MITF**, **MTTP**, **MX1**, **NROB2**, **NUCB2**, **PLA2G7**, PLA2G15, PLCD1, **PLIN2**, **PPARA**, SAT1, SGK1, **SUCLG2**, **STS**, **TMEM55A**, TTR
Small molecule Biochemistry	9.38E-06-3.46E-02	**AADAC**, ABCB1, AKR1D1, **ALB**, **ALDH8A1**, AOC3, **AP2M1**, **APOH**, ATP11B, **BBOX1**, CALB1, **CD36**, **CHKA**, CLPS, **CYP1A1**, **CYP2C9**, CYP2C18, , DIO3, **DLD**, DPYS, **EIF6**, **EPHX2**, **FGF19**, **GCG**, **Gsta4**, **HLF**, **HMGCS2**, **IL8**, **LGALS8**, **MITF**, **MTTP**, **MX1**, **NROB2**, **NUCB2**, **PLAG7**, PLA2G15, PLCD1, **PLIN2**, **PPARA**, SAT1, SGK1, **SLC25A15**, **STS**, **SUCLG2**, **TMEM55A**, TTR, **UGDH**,
Molecular Transport	3.78E-04-3.46E-02	**AADAC**, ABCB1, **ALB**, **ALDH8A1**, AOC3, **AP2M1**, **APOH**, ATP11B, CALB1, **CD36**, **CHKA**, CUBN, DIO3, **EG667604**, **EPHX2**, **ERP29**, **FGF19**, **GCG**, **GDI2**, **HBE1**, **HERPUD1**, **HLA-C**, HLA-DMA, **IL8**, KCNJ15, **LGALS8**, **MCL1**, **MT3**, **MTTP**, **NROB2**, **NUCB2**, PLCD1, **PLIN2**, **PPARA**, **RAB27A**, SAT1, SGK1, SLC23A2, **SLC25A15**, **SLC31A1**, **SNX13**, **SRP54**, **STEAP1**, **STXBP3**, TTR
Cell Death	4.07E-04-3.32E-02	ABCB1, **ALB**, **ATXN3**, CALB1, **CHKA**, **CTTN**, **CYP2C9**, DIO3, **DLST**, DNASE1, **FBLN1**, **FGF19**, **GCG**, **HERPUD1**, **HLA-C**, HLA-DRB4, **HLF**, **IL8**, **IL7R**, **LGALS8**, **MAP2K4**, **MCL1**, MELK, **MME**, **MT3**, **NROB2**, **OSGIN1**, **P4HB**, PCSK2, PHB, **PKN2**, **PLA2G7**, **RASD1**, **RINT1**, SAT1, **SERPINB10**, SGK1, **TPD52**, TTR, **USP18**
Protein Synthesis	4.07E-04 2.80E-02	**ATXN3**, CPA2, **CYP1A1**, **DLD**, **DTL**, **FARSB**, **GCG**, **HERPUD1**, **HLA-C**, **MME**, **MTTP**, PCSK2, **PPARA**, **SERPINB10**, **SERPINE2**, **STX12**, **TMPRESS7**, **USP18**

[1]The genes included in this analysis were differentially expressed between LRFI and HRFI by microarray analysis. The genes up-regulated in LRFI are in bold and all other genes were up-regulated in HRFI.

Table 3.4 Comparison of chosen genes differentially expressed between low (LRFI)/high (HRFI) residual feed intake using microarray real-time RT-PCR

Symbol	Name	Fold change	
		Microarray	RT-PCR
ALB	Albumin	1.66	3.39
BBOX1	Butyrobetaine (gamma), 2-oxoglutarate dioxygenase	1.53	2.46
FGF19	Fibroblast growth factor 19	1.57	4.61
KCNJ15	Potassium inwardly-rectifying channel, subfamily J, member 15	-1.98	-5.49
LYPLAL1	Lysophopholipase-like 1	-1.29	-1.27
PCSK2	Protein convertase subtilisin/kexin type 2	-1.23	-1.08
PPARA	Peroxisome proliferator activated receptor alpha	1.22	1.22
SAT1	Spermindine/spermine N1-acetyltransferase 1	-1.28	-1.63
STEAP4	STEAP family member 4	-2.81	-2.02
TMEM135	Transmembrane protein 135	1.61	1.12

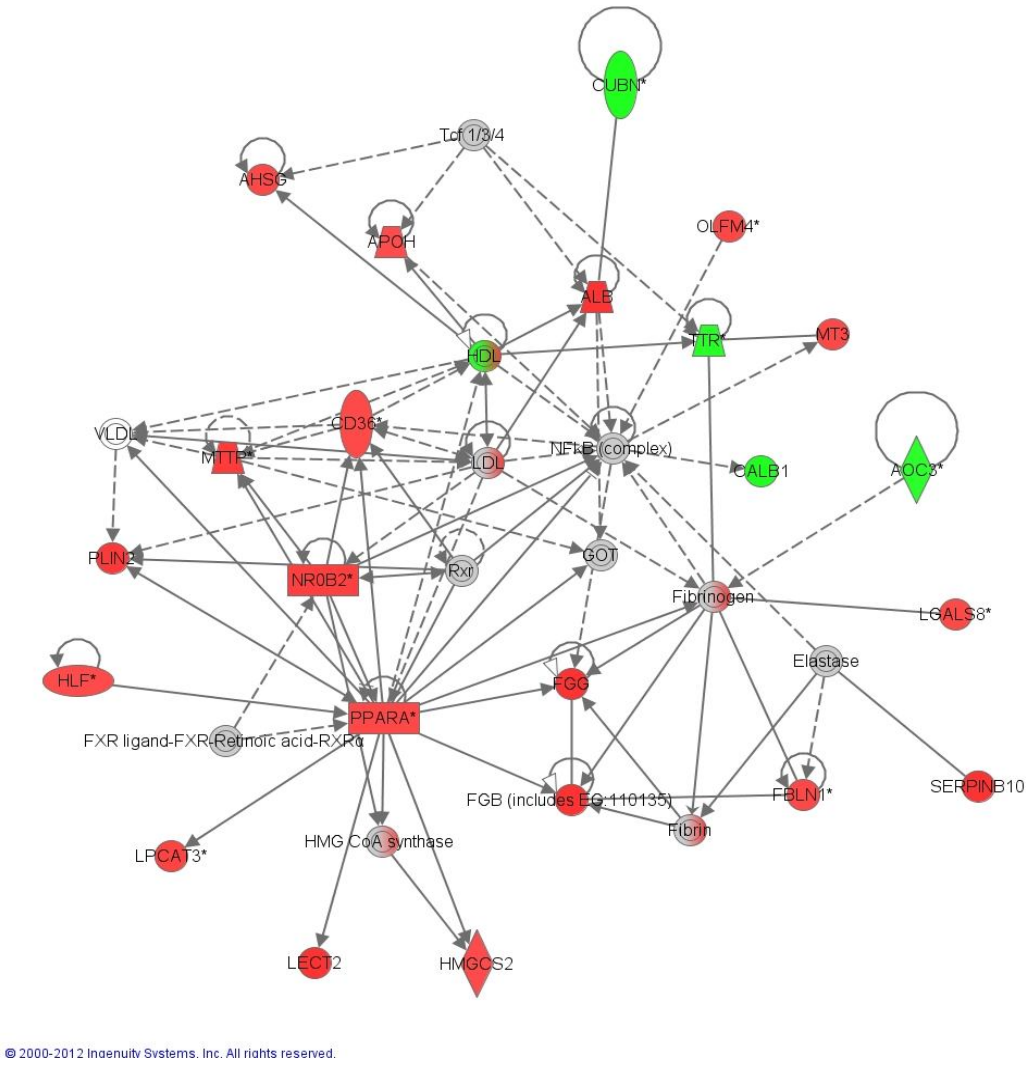

Figure 3.1 Amino acid metabolism and molecular transport network identified through differential expression between low (LRFI) and high (HRFI) residual feed intake chicken lines. The network was generated by Ingenuity Pathway Analysis (IPA, Ingenuity Systems Inc., Redwood City, CA). Gene up-regulated in LRFI and HRFI are shown in red and green, respectively. The genes in the network are: alpha-2-HS-glycoprotein (AHSG); albumin (ALB); amine oxidase; copper containing 3 (AOC3); apolipoprotein H (APOH); calbindin, 28kDa (CALB1); thrombospondin receptor (CD36); cubilin (CUBN); elastase; fibulin 1 (FBLN1); fibrinogen beta chain (FGB includes EG:110135); fibrinogen gamma chain (FGG); fibrin, fibrinogen (FG); FXR ligand-FXR-Retinoic acid; 2-oxoglutarate-glutamate aminotransferase (GOT); high-density lipoprotein (HDL); hepatic leukemia factor (HLF); (S)-3-hydroxy-3 methylglutaryl-CoA acetoacetyl-CoA-lyase (HMG coA synthase); 3-hydroxy-3-methylglutaryl-CoA synthase 3 (mitochondrial) (HMGC2); low density lipoprotein (LDL); leukocyte cell-derived chemotaxin 2 (LECT2); lectin, galactoside-binding, soluble, 8 (LGALS8); lysophosphatidylcholine acyltransferase 3 (LPCAT3); metallothionein 3 (MT3), microsomal triglyceride transfer protein (MTTP), NF KAPPA B (NFkB); nuclear receptor subfamily 0, group B, member 2 (NROB2); olfactomedin 4 (OLFM4); perilipin 2 (PLIN2); peroxisome proliferator-activated receptor alpha (PPARA); retinoid receptor (Rxr); serpin peptidate inhibitor, clad B,member 10 (SERPINB10); Tcf 1/3/4; transthyretin (TTR); and very low density lipoprotein (VDLD).

CHAPTER 4

Differential expression of genes in the avian mechanistic target of rapamycin (avTOR) pathway in a divergent line selected for feed efficiency in meat-type chickens[1]

[1] Lee, J. and S. Aggrey (2012) To be submitted to the journal of genetics.

ABSTRACT

The mechanistic target of rapamycin (mTOR) is a highly conserved serine-threonine kinase that serves as intracellular energy and nutrient sensor and regulates cell division, growth, survival and apoptosis. The relationship of mTOR in mediating feed intake and growth in poultry is unknown. The objective of this study was to investigate avian TOR signaling activities in peripheral tissues in chickens divergently selected for low (LRFI) or high (HRFI) residual feed intake. The differential expression of genes in the avian TOR pathway was assayed using real-time quantitative PCR. The assays were performed in duodenal and liver tissues at day 35 and 42. Plasma insulin, insulin-like growth factor I and II were also assayed at the same time period. In the duodenum mTOR was upregulated in the LRFI at both ages compared to the HRFI. Other genes differentially expressed at day 35 include AKT, EEF2, EIF4EBP1, PDK1, RPS6KP1, MLST8, GHRL, PI3K, FOXO1 and MDM2. At day 42, there was no change in expression of mTOR target RPS6KP1, and also MDM2. In the liver, changes in mTOR genes primarily occurred at day 42. There were also differences in plasma insulin and IGFI levels at day 35 between the LRFI and HRFI, but no such differences at day 42. In the duodenum at day 35, the PI3K/mTORC1 pathways are putatively activated with differential autophagy and apotosis in the LRFI line whereas at day 42, much of the regulation in the duodenum occurs through the AKT/mTORC2 pathways. In the liver, expression differences suggest mTORC1 control at day 42. We hypothesize that there could be nutrient deprivation in the duodenum cells of the LRFI line at day 35 resulting from active transport of amino acids from the duodenum rather than from starvation since all birds from both the low and high feed efficient lines were fed *ad-libitum* on the same diet

INTRODUCTION

The mechanistic target of rapamycin (mTOR) is a highly conserved serine-threonine kinase that serves as intracellular ATP sensor (Dennis et al. 2001). mTOR activity is regulated by hormones (including insulin/insulin-like growth factor), nutrients (including amino acids) and mitogens to stimulate protein synthesis and cell proliferation (Cota et al., 2006; Avruch et al., 2006). The mTOR signaling pathway in the hypothalamus is activated by positive energy status characterized by elevated ATP/AMP ratio (Dennis et al., 2001). However, upon depletion of ATP, activation of AMP-activated protein kinase (AMPK) inhibits mTOR signaling, thereby suppressing protein synthesis which is the process by which AMPK conserve cellular energy during low energy state (Tokunaga et al., 2004). However, administration of specific nutrients such as the branched chain amino acid leucine also activates mTOR signaling that leads to decreases in food intake and body weight (Cota et al., 2006). mTOR phosphorylates and regulates several of its downstream effectors involved in the control of protein translation, including p70 ribosomal S6 kinase (p70S6K), eukaryotic initiation factor 4E-binding protein 1 (4E-BP1) and eukaryotic initiation factor 4G (eIF4G) (Tokunaga et al., 2004) and this effect is mediated through the tuberous sclerosis complex (TSC1/2) and Ras homolog enriched in brain (Rheb) (McManus and Allesi, 2002; Tokunaga et al., 2004). Thus, mTOR coordinate intracellular energy and amino acids pathways.

There are two mTOR complexes; mTOR complex 1 (mTORC1) is responsible for nutrient sensing activities and is comprised of mTOR, G protein-subunit-like protein, mLST8, and regulatory-associated protein of TOR (raptor), whereas mTORC2 phosphorylates Akt protein kinase B and comprise of mTOR and rapamycin-insensitive companion of mTOR (Rictor), GβL and stress activated protein kinase interacting protein 1 (SIN1) (Yang and Guan,

2007). The raptor–mTOR pathway regulates cell growth (accumulation of cell mass), and the two best characterized downstream effectors of mTORC1 are S6K1 and eIF4E-BP1 (Yang et al., 2008). The rapamycin-insensitive rictor–mTOR pathway regulates Akt/PKB, PKCa, Rho/Rac to control cell survival, proliferation, metabolism and the cytoskeleton. Akt/PKB (protein kinase B) is a key component of the insulin/PI3K signaling pathway and modulates cell survival and proliferation (Frias et al., 2006). Huang et al. (2008) suggested that the TSC/2 complex positively regulates mTORC2 in a manner independent of its GTPase-activating protein towards Rheb, and that the TSC1/2 complex can physically associate with mTORC2, but not mTORC1 demonstrating that TSC1/2 complex inhibits mTORC1 and activates mTOR2, which promotes Akt activation through different mechanisms.

The mTOR signaling pathway is co-localized with AgrP/NPY and proopiomelanocortin neurons in arcuate hypothala, but, significant elevation of mTOR signaling was observed in liver and skeletal muscle of insulin-resistant obese rats fed on high fat diet (Khamzina et al., 2005) suggesting peripheral cellular mTOR signaling. Xu et al. (2009) further demonstrated that the mTOR signaling system in gastric endocrine cells functions as a peripheral fuel sensor to alter the expression of ghrelin which in turn on hypothalamic neurons to regulate food intake. The AMPK also functions in peripheral tissues such as liver and skeletal muscle to bring about changes in energy balance (Carling, 2004; Kahn et al., 2005). Thus, the coordinated regulation of hypothalamic AMPK and its downstream action on metabolic pathways plays a critical role in integrating hormonal and nutrient signaling that affects feed intake and whole-body energy homeostasis (Xue and Kahn, 2006). The objective of this study was to investigate avian TOR signaling activities in peripheral tissues (duodenum and liver) in chickens, and ascertain how the TOR signaling pathway affects feed efficiency.

MATERIALS AND METHODS

Experimental Population

We used duodenum and liver tissues from a chicken population that is divergently selected for residual feed efficiency (RFI). The birds were selected on low or high at day 35-42 based on RFI breeding values. Chicks were sexed at hatched and placed in pens with litter and fed a ration containing 225 g/kg protein, 52.8 g/kg fat, 25.3 g/kg fiber, 12.90 MJ ME/kg, 9.5 g/kg calcium (Ca), and 7.2 g/kg total phosphorus (P) (4.5 g/kg available P) until 18 d of age. Hereafter, they were fed 205 g/kg protein, 57.6 g/kg fat, 25.0 g/kg fiber, 13.20 MJ ME/kg, 9.0 g/kg Ca and 6.7 g/kg total P. (4.1 g/kg available P). At 28 d, birds were fasted for 12 hours and transferred to individual metabolism cages until 42 days of age. Birds were kept on a 14L:10D light regimen. The feed intake, RFI and body weight gain were taken for days 28-35 and 35-42 and are presented in Table 4.1. Tissues were collected from 4 males of each line at days 35 and 42. All animal protocols were approved by the Animal Care and Use committee of the University of Georgia.

RNA extraction and real time PCR analysis

Total RNA were extracted from duodenum and liver tissues using Trizol reagent (Invitrogen Corp., Carlsbad, CA, USA) according to manufacturer's instructions. For cDNA synthesis, 2 µg of total RNA was reversed transcribed with high capacity cDNA reverse transcription kits according to manufacturer's protocol (Applied Biosystems, Carlsbad, CA, USA). Real-time PCR reactions were performed using the StepOnePlus (Applied Biosysems, Carlsbad, CA). 0.5µl of cDNA served as a template in a 20µl PCR mixture containing 0.3µl each of forward and reverse primers from 10 µM stocks and 2X Fast SYBR Green Master Mix

(Applied Biosystems, Carlsbad, CA). The PCR conditions were 95°C for 20 seconds, followed by 40 cycles of 95°C for 3 seconds and 60°C for 30 seconds. In addition, at the end of each reaction, a melting temperature curve of every PCR reaction was determined. Data were analyzed according to $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001) and were normalized by β-actin expression in each sample. Differential mRNA expression was expressed as LRFI/HRFI. The NCBI accession numbers, forward and reverse primers, and annealing temperatures of genes used in this study are provided in Table 4.2.

Hormonal levels

About 4 mL of blood was collected from each bird at day 35 and 42 using EDTA as an anticoagulant. The blood samples were centrifuged for 10 min at 503 × *g*, and the plasma was separated and stored frozen at −20°C. The frozen plasma was analyzed in duplicate for IGF-I, IGF-II and insulin using homologous hormone assays. To avoid interassay variation, all samples were analyzed within one assay. Double-antibody radioimmunoassay were used to determine plasma concentrations of IGF-I with an intraassay CV of 2.8% (McMurtry et al., 1994), chicken IGF-II with an intraassay CV of 3.7% (McMurtry et al., 1998), insulin with an intraassay CV of 2.2% (McMurtry et al., 1983).

RESULTS

The feed efficiency and physiological parameters of the LRFI and HRFI lines are provided in Table 4.1. The divergent selection was based on day 35-42 RFI. The LRFI line improved feed efficiency by reducing feed intake (FI) compared to the feed inefficient HRFI line at that age period. However, for the correlated response at day 28-35, the LRFI line improved

feed efficiency by reducing both FI and BWG. Insulin and IGFI levels were lower ($P \leq 0.01$), and IGFII was higher ($P \leq 0.05$) in the LRFI line at day 35 compared to the HRFI line. There were no differences in insulin, IGFI and IGFII between the two divergently selected lines at day 42. There was an increase in insulin level in both lines from day 35 to 42 and the relative increase was about two fold in the LRFI line compared to the HRFI line. While there was no appreciable change in the IGFI level in both lines from day 35 to 42, there was a significant decline in IGFII levels from day 35 to 42.

The relative levels of mRNA for the genes in the mTOR pathway using duodenum and liver tissues are presented in Tables 4.3 and 4.4, respectively. Genes were considered to be differentially expressed when the $2^{-\Delta\Delta Ct}$ values between the LRFI and HRFI lines were significantly different ($P<0.05$) and had a minimum of 1.2 fold difference. The result suggests that mRNA expression is affected by both age and peripheral tissue. The insulin-like growth factor (IGF)-1 was upregulated at days 35 and 42 in the duodenum in LRFI but was downregulated in the liver for both periods. However, insulin (INS) was downregulated in LRFI at day 35 only in the duodenum, but upregulated in both tissues at day 42. The v-akt murine thymoma viral oncogene homolog (AKT)-3, mTOR associated protein, LST8 homolog (MLST8), AMP-activated protein kinase (AMPK), ghrelin (GHRL) and p53 E3 ubiquitin protein ligase homolog (MDM2) genes were upregulated in HRFI in both duodenum and liver tissues at day 35 however at day 42, AKT3 and MLST8 were downregulated in HFRI in both tissues, whereas AMPK and GHRL were downregulated only in the liver and not the duodenum. The phosphoinositide-3-kinase (PI3K), which is upstream the mTOR complex was downregulated only in LRFI at day 35 only in the duodenum.

The mTOR gene was upregulated in the duodenum of LRFI at both ages, but only at day 42 in the liver. In the duodenum, the RPTOR independent companion of mTORC2 (RICTOR) and 3-phosphoinositide dependent protein kinase -1 (PDPK1) were downregulated in LRFI at day 35 and upregulated in day 42, but in the liver PDPK1 was upregulated only at day 42 and not day 35. Among the genes downstream the mTORC1 and mTORC2, eukaryotic translation elongation factor 2 (EEF2), eukaryotic translation initiation factor 4E binding protein 1 (EIF4EBP1), foxheard box 01 (FOX01) were downregulated in LRFI at day 35, but upregulated at day 42 in the duodenum. In the liver, EEF2, EIF4EBP1 and FOX01 were not differentially expressed at day 35, but were upregulated in LRFI at day 42.

DISCUSSION

The AMP-activated protein kinase is an evolutionary conserved enzyme that acts a sensor of cellular energy status (Kahn et al., 2005). It modulates energy balance at the whole body level by regulating feed intake and energy expenditure, and peripheral hormones that control feed intake and also modulate the activity of hypothalamic AMPK (Kola, 2008). AMPK was downregulated in the LRFI line at day 35 in both the duodenum and the liver. However, in the duodenum, the downregulation of AMPK at day 35 coincided with an upregulation of mTOR in LRFI. Changes in AMPK expression may affect mTOR differently in different tissues. The direct effect of AMPK on mTOR in the liver and duodenum for the feed efficiency lines is uncertain. If AMPK were to act directly on mTOR, then upregulation of AMPK should lead to a downregulation of mTOR (Gleason et al., 2007), but in the liver at day 42 this inverse relationship did not occur. According to Bang et al. (2012), activation of AMPK down-regulates mTOR signaling and inhibiting AMPK activity increases mTOR signaling. It is also possible that

mTOR is under different controls in different tissues depending on the prevailing cellular energy level in the particular tissue.

In the LRFI line the putative upregulation of mTORC1 in the duodenum at day 35 may be due to rapid transportation of amino acid out of the duodenum. Amino acid deprivation especially leucine results in dephosphorylation of the mTORC1 effectors S6K1 and 4E-BP1 (Hay and Sonenbert, 2004). There was downregulation of both S6K1 and 4E-BP1 in the duodenum at day 35 in the LRFI line, but that cannot be directly attributed to leucine deprivation. In experimentally induced rats, leucine administration alone had no effect on mTOR signally to 4E-BP1 or S6k1, but leucine administered in combination with insulin infusion enhanced mTOR signaling to 4E-BP1 and S6K1 (Anthony et al., 2002). Both INS and PI3K were downregulated in the LRFI lines in the duodenum at day 35 suggesting that the PI3K pathway was active in relation to feed efficiency especially when both PI3K and mTOR pathways converge on common downstream targets; the S6Ks and 4E-BPs (Richardson, et al., 2004). However, it is unlikely that S6K1 in the duodenum at day 35 is under the direct control of mTORC1. The level of plasma insulin at day 35 was significantly lower in the LRFI line compared to the HRFI line. Plasma insulin levels increased at day 42, which were in concordance with expression. However, we cannot establish the direct relationship between plasma insulin levels and INS expression. In peripheral tissues, insulin signaling involves activation of the insulin receptor substrate (IRS)-PI3K enzyme system (Niswender et al, 2003). It is therefore possible that S6K1 expression in the duodenum at day 35 is under PI3K pathway control since both insulin and IGFI mediate PI3K activation which has a negative feedback relationship with S6K (Courtney et al., 2010).

S6K1 was upregulated in the duodenum of HRFI at day 35. S6K1 phosphorylates several proteins, including rp6K, elF4B, S6K1 Aly/REF-like target (SKAR) and eukaryotic elongation factor 2 kinase, and affects both initiation and elongation stages of mRNA translation (Kimball and Jefferson, 2006). Upregulation of S6K1 and EEF2 in HRFI at day 35 suggests an increase in the synthesis of components of the protein translation system and potential increase in protein synthesis in the duodenum compared to LRFI. The downregulation of 4E-BP1 in the duodenum of the LRFI line at day 35 suggests increase in autophagy. The mTORC1 regulates translation via S6K1 and 4E-BP (Hay and Sonenberg, 2004). Autophagy is a process where cells degrade cytoplasmic content including organelles and subsequently recycling macromolecules to ensure survival (Wullschleger et al., 2006). Autophagy is a tightly controlled process that plays an important role in cell growth and development that maintains the balance between synthesis, degradation and recycling of cellular products. We hypothesize that there could be nutrient deprivation in the duodenum cells of the LRFI line at day 35 resulting from active transport of amino acids from the duodenum rather than from starvation since all birds from both the low and high feed efficient lines were fed *ad-libitum* on the same diet, but we have no direct evidence to support this hypothesis. However, it has been demonstrated that mTORC1 activation in the hypothalamus suppresses food intake, thereby creating a systemic negative feedback loop to maintain nutrient homeostasis, and mTORC1 also increases nutrient mobilization into peripheral tissues through enhanced lipid storage in adipose tissue (Cota et al., 2007). Therefore, mTORC1 activation in the duodenum may act synergistically with its counterpart in the hypothalamus to suppress feed intake and consequentially increase nutrient mobilization to other peripheral tissues, and in the process activating genes that increase autophagic activity and suppressing

apoptosis to ensure sufficient metabolites to the duodenal cells. It should be noted that the LRFI line improved feed efficiency primarily by reducing feed intake.

Also in the duodenum at day 35, PI3K, AKT2, AKT3, PDK1, FOXO1 and MDM2 were all downregulated whereas AKT1 was upregulated in the LRFI line compared to the HRFI line. The gene expression changes in these genes suggest an involvement of the PI3K/AKT/mTOR pathway in relation to feed efficiency in chickens. The PI3K/AKT/mTOR pathway is known to be involved in metabolism, cell growth and survival, cell-cycle progression, transcription and translation (Fingar and Blenis, 2004). The binding of IGF-1 to its tyrosine kinase receptors results in recruitment of PI3K to the plasma membrane and its activation, which in turn phophorylates several genes including PDK1 and AKT1 in the plasma membrane (Belham et al., 1999). AKT then becomes phosphorylated and activated, which triggers activation of several downstream signaling pathways and consequently increases cell survival, proliferation, and cell growth. (Fingar and Blenis, 2004). AKT activates MDM2 which inhibits apoptosis (Datta et al., 1997). In addition, AKT translocates from the plasma membrane to the nucleus where it phosphorylates FOXO transcription factors (Brunet et al., 1999; Cully et al., 2006). The phosphorylated FOXO proteins then leave the nucleus resulting in a transcriptional program that enhances oxidative phosphorylation for efficient energy production, increased levels of protein folding chaperones, heat shock proteins, and produces antioxidant proteins that decrease the levels of ROS. (Huang and Tindall, 2006; Feng, 2010). Thus, the removal of FOXO from the nucleus reduces the signaling for cellular apoptosis, and drives cells into cell cycle (Burgering and Koops, 2002; Feng, 2010). Gene expression changes at day 35 in the duodenum of the chickens divergently selected for feed efficiency clearly suggest differential cell division, growth, proliferation, autophgy and apoptosis.

The MDM2 gene, is an oncoprotein that plays a role as a regulator of cell proliferation and apoptosis by serving as an important negative regulator of the p53 tumor suppressor (Kruse and Gu, 2009). The PI3K/ATK can activate MDM2 resulting in p53 inhibition. p53 mediates DNA damage-induced cell-cycle arrest, apoptosis, or senescence, and it is controlled by MDM2, which mainly ubiquitinates p53 in the nucleus and promotes p53 nuclear export and degradation. Lai et al. (2010) showed that S6K1 activation is necessary to retain MDM2 in the cytoplasm and to inhibit MDM2-mediated p53 ubiquitination suggesting that S6K is a negative regulator of MDM2 but a positive regulator of p53. Transcriptional changes in MDM2 and S6K1 suggest differential cell division, growth, proliferation and apoptosis in the duodenum of chickens divergently selected for feed efficiency.

The mTORC2 appears to be active in the duodenum at day 42, where mTOR, AKT1, AKT2, AKT3, hypoxia inducible factor 1 (HIF1A), EEF2, EIF4EBP1, PDK1, RICTOR, cyclin D1 (CCND), FOXO1 and glycogen synthetase 3β were all upregulated in LRFI. There were significant changes in IGFI levels between the LRFI and HRFI at day 35, but not day 42, and there was a significant decline in IGFII levels from day 35 to 42 in both lines. High glucose levels lead to the secretion of insulin which results in the production of IGFI. The IGFI/IGFIR/PI3K cascade results in increased concentration of plasma membrane intrinsic protein 3 (PIP3) (Toker and Newton, 2000). Increased PIP3 in turn activates PDK1 and AKT and the plasma membrane. According to Wullschleger et al. (2006), mTORC2 was discovered recently and both upstream and downstream effectors are largely unknown. The mTORC2 also phosphorylates and activates AKT (Sarbossov et al., 2005), and AKT regulates cell proliferation, survival, metabolism and transcription (Zhang et al., 2011). It has been suggested that growth factors may regulate mTORC2 (Jacinto et al., 2004; Sarbassov et al., 2005) but whether this

involves the same signaling cascades that regulate mTORC1 is not yet elucidated. It appears that in the low feed efficiency line, mTORC1 is activated in day 28- 35, whereas AKT/TORC2 is activated in day 35-42 for cell survival and stabilization.

There was no transcription change in the signal transducer and activator of transcription 3 (STAT3) gene in the duodenum at both day 35 and 42. However, in the liver, STAT3 was downregulated in the LRFI line at day35 but upregulated in day 42. STAT3 is a transcription factor which is usually activated in response to cytokines and growth factors (e.g. fibroblast growth factors and epidermal growth factors; Zhang et al., 2005). STAT3 mediates the expression of a variety of genes in response to cell stimuli and plays a role in cell growth and division, cell movement and apoptosis (Hirano et al., 2000). It is possible that stimuli for the duodenum and liver were different and as a result, it led to the difference in the response of STAT3. The vascular endothelial growth factor A (VEGFA) was upregulated in the LRFI line only at day 42 in the duodenum, but in the liver, VEGFA was downregulated at day 35 and upregulated at day 42. VEGFA is a growth factor active in angiogenesis, vasculogenesis and endothelial cell growth which induces endothelial cell proliferation, promotes cell migration and inhibits apoptosis. Brugarolas et al. (2003) demonstrated that TSC2 regulates VEGF through mTOR-dependent and -independent pathways, and that the mTOR independent pathway of VEGF may involve chromatin remodeling. VEGF and insulin which promote angiogenesis, induce Akt-dependant signals. (Shiojima and Walsh, 2002). The upregulation of VEGFA in the LRFI line could support active nutrient transport to other peripheral tissues. The insulin gene was downregulated in the duodenum of the LRFI line at day 35, and upregulated at day 42. However, in the liver, there was no change in INS expression at day 35, but upregulated at day 42.

Activation of PI3K by insulin has been shown to differ depending on the cell type (Xu et al., 2005).

In a mice study, gastric mTORC1 inbition led to upregulation of ghrelin (GHRL) and vice versa (Xu et al., 2009). The mTORC1 and GHRL expression in the duodenum at day 35 supports the conclusion of Xu et al. (200) in mice, but not in day 42, perhaps due to the fact that the mTORC2 is more active at day 42, and not day 35 in relation to feed efficiency. Therefore, the mTORC1 in the duodenum could serve as peripheral energy and/or nutrient gauge to alter the expression of GHRL, which may in turn act on the hypothalamic neurons to regulate feed intake.

In the duodenum, at day 35, the PI3K/mTORC1 pathways are putatively activated with subsequent downregulation of MDM2 and differential autophagy and apotosis in the LRFI line in an effort to support active transport of nutrients to other peripheral tissues. In the liver however, gene expression changes were restricted to STAT3, VEGFA and MDM2 which could be under the control of other pathways not considered in the current study. However, at day 42, much of the regulation in the duodenum occurs through the AKT/mTORC2 pathways, whereas regulation in the liver was putatively controlled by mTORC1. Thus, the PI3K/AKT/mTOR pathway plays a central role in cell growth, autophagy, survival and metabolism to affect feed efficiency for growth in chickens but other signaling pathways cannot be excluded. Genetic markers in key genes in the PI3K/AKT/mTOR pathways could be developed to assist in the improvement of feed efficiency in meat-type chickens. The genetic interrelationships between body weight gain, feed intake and feed efficiency in meat-type chickens have been elucidated (Aggrey et al., 2010). Selecting on RFI at day 35-42 in the population used improved feed efficiency at day 28-35 by reducing feed intake and to a smaller extent body weight gain, and also improved feed efficiency at day 35-42 by reducing feed intake without changes to body

weight gain (Table 4.1). The genetic interrelationships of feed efficiency parameters at day 28-35 are different than those of day 35-42, and that could also explain the changes in gene expression at the two age periods.

Table 4.1 Means (±SE) of feed efficiency and physiological parameters in a chicken population divergently selected for low (LRFI) or high (HRFI) residual feed intake

Trait	Period	LRFI	HRFI	Pr>F
MBW	28	171.43±1.15	173.99± 1.27	0.1357
BWG	28-35	394.37±4.90	420.89± 5.42	0.0003
FI	28-35	707.89±8.65	803.15± 9.58	<0.0001
RFI	28-35	-31.86±6.38	32.33± 7.07	<0.0001
FCR	28-35	1.80±0.02	1.93± 0.02	<0.0001
MBW	35	222.20±1.47	227.81± 1.62	0.0103
BWG	35-42	376.39±4.72	372.09± 5.23	0.5406
FI	35-42	776.60±9.96	886.72±10.91	<0.0001
RFI	35-42	-41.35±8.33	54.80± 9.22	<0.0001
FCR	35-42	2.13±0.04	2.49± 0.05	<0.0001
INS (pg/ml)	35	0.78±0.03	0.92± 0.03	0.0021
IGFI (ng/ml)	35	36.76±0.86	41.31± 0.85	0.0002
IGFII (ng/ml)	35	101.26±4.23	89.51± 4.16	0.0485
INS (pg/ml)	42	1.19±0.03	1.22± 0.03	0.4635
IGFI (ng/ml)	42	39.62±0.78	40.73± 0.83	0.3316
IGFII (ng/ml)	42	61.86±2.37	58.59± 2.50	0.3436

[1]MBW = Metabolic body weight (BW); BWG = BW gain; FI = feed intake; FCR = feed conversion ratio; RFI = residual feed intake; INS=insulin; IGF=insulin-like growth factor
[2]All traits measured in (g) except for FCR which was (g/g);
[3]Age (d) or age range (d) that trait was measured

Table 4.2 Primer sequences used in quantitative RT-PCR assay and size of product

[1]Gene Symbol	GenBank Accession No.	Forward Primer	Reverse Primer	Size Bp
AKT1	NM_205055.1	AGT GAA GGA AGG ATG GCT CCA CAA	TTC ATC AGC TGG CAC TGA GCT ACT	174
AKT2	AF181260.1	TCA GAG ATC GAC ACG CGG TAC TTT	ATG CTG GCA GAG TAT GAG AAC TGG	143
AKT3	XM_419544.2	CAG AGC GAC CAA AGC CAA ACA CAT	TCT GCT ACA GCC TGG ATT GCT TCT	130
EEF2	NM_205368.1	AGC CAA TCC AAA GGA CCA TCC TCA	ACT GAT CAA CAC CAA CCA GAC CGA	99
EIF4EBP1	XM_424384.2	ATG GAG TGC CGT AAT TCT CCG GTT	ACT CCT CCA CAA TTG GGC TGG TAA	88
HIF1A	NM_204297.1	AGG CAT CCA TTA TGA GGC TGA CCA	AGC TCC TTC TCC ATG TTG GCT TCT	97
IGF1	NM_001004384.2	AAA GCC ACC TAA ATC TGC ACG CTC	AGT ACC CTG CAG ATG GCA CAT TCA	178
mTOR	XM_417614.2	TTG GGT TTG CTT TCT GTG GCT GTC	ACA GAC TTC TGC CTC TTG TGA GCA	119
PDPK1	NM_001012529.1	AGC AGA TTC CAG CAC TCC ATC TGT	TCA GGT CGT TTC TTT CGA GGC TGT	153
RICTOR	XR_027072.1	GCA CAT TGC ACA TTT CGA CTC CCA	TAG CCT CCG CTT CTT CAT GCA TCT	94
RPS6	NM_205225.1	CTC GTG TTC TGC AAC ATA AGC GCA	AAT CTG CTG CCT CCT CCT TGT TCT	81
RPS6KP1	NM_001030721.1	TTT GCC TCC CTA CCT CAC ACA AGA	AAG AAC GGG TGA GCC TGA ACT TCT	123
RPTOR	XM_426232.2	AGA ACT TTG ACT CAG CCA GGT GGT	CGT TCA GCT GAG TTG CCC ACA AAT	142
STAT3	NM_001030931.1	ACT GCT GTC AGC CAT GGA GTA TGT	TTC TTG ATC TGC TGC CGA GTC TGT	180
VEGFA	NM_205042.2	AAA GAG AAA GCG CAA GAA AGG CCG	TCG ACT TGC AAC GTG AGT CTG TGA	143
IRS1	NM_001031570.1	TTG GTA TCT ACC GCC TTT GCC TGA	AAG AAG TTC TCG GAG TGT CCG CAA	124
INS	NM_205222.2	CCA GCA CCT CTG TGG CTC CCA	GGG CTG CTC GAC ATC CCG TC	103
MLST8	XM_414858.2	TGC CAG CGG ATC TTC CAA GTG AAT	TGT GCA CAG AGT TCA CGG AGA CTT	172
AMPK	NM_001039603.1	AGG TGT AAG AAG AGC GAA GTG GCA	TCT TCC GTC GAA CAC GCA AGT AGT	146
GHRL	AY303688.1	AAA GCA GCC AGC AAT GGC TCT AAC	TTC TGT GCC TCG GCG ATG TAA TCT	179
CCND	NM_205381.1	CAA ATG GAG CTG CTG CTG GTG AAT	TGA TCT GTT TGG TGT CCT CTG CCA	118
PI3K	AF001076.1	AGA GAG CGT GTG CCC TTT GTC TTA	TCC TGA GCC AAG CAT CAT GGA GAA	183
FOXO1	NM_204328.1	AGT GCA GAA TGA GGG AAC AGG GAA	TGA GAT CCA GGG CTG TCA CCA TTT	198
MDM2	NM_001199384.1	TCG GAC AGC ATC TCG TTG ACC TTT	TGG TCT GAA ACA GAA CCG TGG TCA	176
GSK3B	XM_416557.3	AAA CGT CAA GTT ACC AAA CGG GCG	TCT GAG GCT GCT GTA GCG TTT GTA	159
β-actin	NM_205518.1	AGA CAT CAG GGT GTG ATG GTT GGT	TCC CAG TTG GTG ACA ATA CCG TGT	125

[1]The genes are: v-akt murine thymoma viral oncogene homolog 1 (AKT1); v-akt murine thymoma viral oncogene homolog 2 (AKT2); v-akt murine thymoma viral oncogene homolog 3 (AKT3); eukaryotic translation elongation factor 2 (EEF2); eukaryotic translation initiation factor 4E binding protein 1 (EIF4EBP1); hypoxia inducible factor 1 (HIF1A); insulin-like growth factor 1 (IGF1); mechanistic target of rapamycin (mTOR); 3-phosphoinositide dependent protein kinase-1 (PDPK1); RPTOR independent companion of MTOR, complex 2 (RICTOR); ribosomal protein S6 (RPS6); ribosomal protein S6 kinase, 70kDa, polypeptide 1 (RPS6KP1); regulatory associated protein of MTOR, complex 1 (RPTOR); signal transducer and activator of transcription 3 (STAT3); vascular endothelial growth factor A (VEGFA); insulin receptor substrate 1 (IRS1); Insulin (INS); MTOR associated protein, LST8 homolog (MLST8); AMP-activated protein kinase (AMPK); ghrelin (GHRL); cyclin D1 (CCND); phosphoinositide-3-kinase (PI3K); forkheard box 01 (FOXO1); p53 E3 ubiquitin protein ligase homolog (MDM2); glycogen synthase 3 beta (GSK3B); beta-actin (β-actin)

Table 4.3 Duodenum mRNA expression of genes in the avTOR pathway in chickens divergently selected for residual feed efficiency.

Gene Symbol	Gene Name	Fold Change	
		Day 35	Day 42
AKT1	v-akt murine thymoma viral oncogene homolog 1	1.35	1.39
AKT2	v-akt murine thymoma viral oncogene homolog 2	-1.28	1.20
AKT3	v-akt murine thymoma viral oncogene homolog 3	-1.72	1.73
EEF2	eukaryotic translation elongation factor 2	-1.56	1.37
EIF4EBP1	eukaryotic translation initiation factor 4E binding protein 1	-2.17	1.44
HIF1A	hypoxia inducible factor 1	1.03	1.49
IGF1	insulin-like growth factor 1	1.44	1.34
mTOR	mechanistic target of rapamycin	1.21	1.35
PDPK1	3-phosphoinositide dependent protein kinase-1	-1.39	1.40
RICTOR	RPTOR independent companion of MTOR, complex 2	-1.25	1.83
RPS6	ribosomal protein S6	-1.45	1.21
RPS6KP1	ribosomal protein S6 kinase, 70kDa, polypeptide 1	-1.56	1.12
RPTOR	regulatory associated protein of MTOR, complex 1	-1.04	1.43
STAT3	signal transducer and activator of transcription 3	-1.07	-1.08
VEGFA	vascular endothelial growth factor A	1.09	1.26
IRS1	insulin receptor substrate 1	1.12	1.31
INS	Insulin	-2.70	3.02
MLST8	MTOR associated protein, LST8 homolog	-1.30	1.30
AMPK	AMP-activated protein kinase	-1.25	1.17
GHRL	Ghrelin	-2.70	1.06
CCND	cyclin D1	-1.10	1.20
PI3K	phosphoinositide-3-kinase	-1.25	1.12
FOXO1	forkheard box 01	-1.61	1.61
MDM2	p53 E3 ubiquitin protein ligase homolog	-1.33	1.11
GSK3B	Glycogen synthase 3 beta	-1.04	1.24

Table 4.4 Liver mRNA expression of genes in the avTOR pathway in chickens divergently selected for residual feed efficiency.

Gene Symbol	Gene Name	Fold Change	
		Day 35	Day 42
AKT1	v-akt murine thymoma viral oncogene homolog 1	1.05	1.16
AKT2	v-akt murine thymoma viral oncogene homolog 2	-1.01	1.68
AKT3	v-akt murine thymoma viral oncogene homolog 3	-1.32	1.29
EEF2	eukaryotic translation elongation factor 2	1.04	1.32
EIF4EBP1	eukaryotic translation initiation factor 4E binding protein 1	-1.05	1.39
HIF1A	hypoxia inducible factor 1	1.01	1.18
IGF1	insulin-like growth factor 1	-1.30	-1.27
mTOR	mechanistic target of rapamycin	1.00	1.22
PDPK1	3-phosphoinositide dependent protein kinase-1	1.09	1.30
RICTOR	RPTOR independent companion of MTOR, complex 2	-1.10	-1.14
RPS6	ribosomal protein S6	1.18	1.15
RPS6KP1	ribosomal protein S6 kinase, 70kDa, polypeptide 1	1.01	1.39
RPTOR	regulatory associated protein of MTOR, complex 1	1.10	1.13
STAT3	signal transducer and activator of transcription 3	-1.32	1.75
VEGFA	vascular endothelial growth factor A	-1.56	1.20
IRS1	insulin receptor substrate 1	-1.33	-1.10
INS	insulin	-1.02	1.28
MLST8	MTOR associated protein, LST8 homolog	-1.39	1.28
AMPK	AMP-activated protein kinase	-1.33	1.33
GHRL	ghrelin	-1.69	1.62
CCND	cyclin D1	1.09	1.12
PI3K	phosphoinositide-3-kinase	1.08	1.10
FOXO1	forkheard box 01	1.19	1.44
MDM2	p53 E3 ubiquitin protein ligase homolog	-1.39	1.11
GSK3B	Glycogen synthase 3 beta	-1.10	1.10

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CHAPTER 5

Transcriptomic analysis of genes in the Nitrogen recycling pathway of meat-type chickens divergently selected for feed efficiency[1]

[1] Lee, J. and S. Aggrey (2012) To be submitted to the journal related to genetics

ABSTRACT

Understanding of the dynamics of ammonia detoxification and excretion in uricotelic species is lagging behind ureotelic species. The relationship between feed intake, feed efficiency, weight gain, and nitrogen excretion in chickens is unknown. The objective of this study was to investigate the transcriptomics differences in key genes in the nitrogen (N) metabolism and purine pathway in a chicken population divergently selected for low (LRFI) or high (HRFI) residual feed intake at two different ages. The differential expression of genes in the N recycling and purine biosynthesis pathways were assayed using real-time PCR. The assays were performed in duodenum, liver, *Pectoralis major* (*P. major*), and kidney tissues at day 35 and 42. Fecal nitrogen was also determined at day 42. The *P major* was the major source of glutamine in LRFI at day 28-35. Also in LRFI, ALDH4A1, CCBL1, GLS, GLUD1, GOT1, GOT2 and PPAT were down-regulated in all 4 tissues while ASNS and GLS were up-regulated in the muscle. At day 42, ALDH4A1, GOT2 and GS were up-regulated in the muscle of LRFI. The ALDH4A1, ASNS, GLUD1, GOT1, GOT2, GS and PPAT genes were up-regulated in LRFI compared to HRFI. There was a significant positive correlation between RFI and fecal N. Gene expression changes in GLS and GS at day 28-35 suggested a reduced rate of protein synthesis in LRFI, which may reflect the decline in weight gain compared in HRFI. However, at day 35-42, gene expression differences between the chicken lines suggested that the putative reduction in deamination and the up-regulation of PPAT in the liver and kidney of LRFI relative to HRFI may be due to feedback regulation in response to purine nucleotides needed to maintain weight gain.

Key Words: Nitrogen cycling, protein, purine biosynthesis, uric acid, feed efficiency

INTRODUCTION

The world will need 70-100% more food by 2050 (Godfray et al, 2010) when the global population is expected to reach 9 billion. This global demand for food will lead to competition for agricultural inputs and create an urgent need to curtail the impact of intensified food production on the environment (Royal Society, 2009), health and wellbeing of animals, a concept called intensive sustainability. The efficiency of feed utilization in meat-type chicken has not caught up with its growth potential. Since feed costs constitute about 60-70% of the total production cost and feed is a limited commodity, improvements in feed efficiency will allow for greater meat production while reducing the amount of feed required for growth, thereby reducing production costs and negative environmental impacts. The major components of feed are protein, energy, calcium and phosphorus. The retentions of protein and P are 50-70 and 40%, respectively leading to substantial amounts of nitrogen (N) and phosphorus (P) in poultry waste. Excreta N can be converted to ammonia and nitrates. Nitrate in water is linked to methemoglobinemia in infants, toxicity in livestock, and eutrophication in both fresh and saline waters (Sharpley et al., 1998). Pollution of surface water by P restricts its use for fisheries, recreation, and drinking due to increased growth of undesirable algae, and oxygen shortages caused by their senescence and decomposition (Kotak et al., 1993).

Therefore, improvements in feed efficiency, especially efficiency of protein use will improve productivity and simultaneously reduce the amount of manure N. Feeding high protein levels improves feed efficiency, but this approach is not sustainable as it also substantially increases the amount of fecal N (Baeza et al., 2012). Current genetic improvement methods are also limited in the rate of annual genetic improvement because the actual feed efficiency phenotypes defined by genotypes or gene markers are not known. Transcriptomics is a critical

step in understanding the mechanism that underlies the interaction of nutrition and the genome. Chicken populations are usually fed on the same diet therefore genomic differences on how chicken populations interact with the same diet will provide the gene network that pertains to nutrient utilization. However, there is very scant information on the molecular aspects of protein catabolism and N recycling in species that excrete N in the form of uric acid including chickens.

The objective of this study was to investigate the transcriptomics differences in key genes in the N metabolism and purine pathway in a chicken population divergently selected for low (LRFI) or high (HRFI) residual feed intake at two different ages using the duodenum, *Pectoralis major* (*P. major*), liver and kidney tissues. Aggrey et al. (2010) have shown that RFI at day 28-35 is genetically correlated to BWG whereas RFI at day 35-42 is not correlated to BWG. These two ages would potentially delineate the underlying mechanisms of N recycling at different stages of growth in the meat-type chicken.

MATERIALS AND METHODS

Experimental Population

We used duodenum, liver, *P. major* (major breast muscle), and kidney tissues from a chicken population that is divergently selected for residual feed efficiency (RFI). The birds were selected on low or high at day 35-42 based on RFI breeding values. Chicks were sexed at hatched and placed in pens with litter and fed a ration containing 225 g/kg protein, 52.8 g/kg fat, 25.3 g/kg fiber, 12.90 MJ ME/kg, 9.5 g/kg calcium (Ca), and 7.2 g/kg total phosphorus (P) (4.5 g/kg available P) until 18 d of age. Hereafter, they were fed 205 g/kg protein, 57.6 g/kg fat, 25.0 g/kg fiber, 13.20 MJ ME/kg, 9.0 g/kg Ca and 6.7 g/kg total P (4.1 g/kg available P). At 28 d, birds were fasted for 12 hours and transferred to individual metabolism cages until 42 days of

age. Birds were kept on a 14L:10D light regimen. The feed intake, RFI and body weight gain were taken for days 28-35 and 35-42 and are presented in Table 5.1. We randomly selected 100 birds from both HRFI and LRFI and determined the fecal nitrogen (FN) using the method described by AOAC (1995). We estimated the correlation coefficient between RFI and FN, and FN and N intake (NI) using PROC CORR procedure (SAS, 1998). The NI was estimated from the amount of N in the feed and the amount of feed consumed. All animal protocols were approved by the Animal Care and Use committee of the University of Georgia.

RNA extraction and real time PCR analysis

Total RNA were extracted from duodenum, breast, liver and kidney tissues using Trizol reagent (Invitrogen Corp., Carlsbad, CA, USA) according to manufacturer's instructions. For cDNA synthesis, 2 µg of total RNA was reversed transcribed with high capacity cDNA reverse transcription kits according to manufacturer's protocol (Applied Biosystems, Carlsbad, CA, USA). Real-time PCR reactions were performed using the StepOnePlus (Applied Biosysems, Carlsbad, CA). 0.5µl of cDNA served as a template in a 20µl PCR mixture containing 0.3µl each of forward and reverse primers from 10 µM stocks and 2X Fast SYBR Green Master Mix (Applied Biosystems, Carlsbad, CA). The PCR conditions were 95°C for 20 seconds, followed by 40 cycles of 95°C for 3 seconds and 60°C for 30 seconds. In addition, at the end of each reaction, a melting temperature curve of every PCR reaction was determined. Data were analyzed according to $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001) and were normalized by β-actin expression in each sample. Differential mRNA expression was expressed as LRFI/HRFI. The NCBI accession numbers, forward and reverse primers, and annealing temperatures of genes used in this study are provided in Table 5.2.

RESULTS

The feed efficiency parameters of the LRFI and HRFI lines are provided in Table 5.2. The divergent selection was based on day 35-42 RFI. The LRFI line improved feed efficiency by reducing feed intake (FI) compared to the feed inefficient HRFI line at day 35-42. However, for the correlated response at day 28-35, LRFI improved feed efficiency by reducing both FI and BWG. The correlation between RFI and FN was 0.45 ($P \leq 0.001$), and that between NI and FN was 0.33 ($P \leq 0.001$). Abdominal fat yield was different between LRFI and HRFI. LRFI had a lower abdominal fat yield ($P \leq 0.09$) compared to HRFI.

The relative mRNA levels of aldehyde dehydrogenase 4, member A1 (ALDH4A1), asparagine synthetase (ASNS), cystein conjugate-beta lyase 1 (CCBL1), glycine amidinotransferase (GATM), glutaminase (GLS), glutamate dehydrogenase 1 (GLUD1), glutamic-oxaloacetate transaminase (GOT) 1, GOT2, glutamine synthetase (GS), and phosphoribosyl pyrophosphate amidotransferase (PPAT) in the duodenum, breast, liver and kidney tissues at days 28 and 35 are presented in Tables 5.3 and 5.4, respectively. Genes were considered to be differentially expressed when the $2^{-\Delta\Delta Ct}$ values between LRFI/LRFI had a minimum of 1.2 fold difference. There were differences in gene expression between tissues and also between ages. ALDH4A was down-regulated in the duodenum, muscle and kidney at day 28-35 but up-regulated in the muscle, liver and kidney at day 35-42. GS was down-regulated in the duodenum but up-regulated at day 28-35 but at day 35-42, GS was up-regulated in the muscle, liver and kidney. The expression of GLS was opposite between day 28-35 and 35-42 for each of the 4 tissues investigated. The PPAT gene was down-regulated in the duodenum and kidney at day 28-35. However, at day 35-42 the gene was down-regulated in the muscle, and up-regulated in the liver and kidney tissues. Interestingly, both GOT1 and GOT2 were down-

regulated in all tissues at day 28-35, but at day 35-42, GOT1 was up-regulated only in the liver and kidney whereas GOT2 was down-regulated in the duodenum and up-regulated in the muscle, liver and kidney.

DISCUSSION

Dietary protein is metabolized to provide amino acids for growth and various physiological processes in the body. The by-product of amino acid catabolism is ammonia which is toxic and in avian species eliminated as uric acid (Milroy, 1903). Ammonia is also produced endogenously mainly in skeletal muscles, kidney and the brain (Spanaki and Plaitakis, 2012). Elevated levels of ammonia changes the properties of the blood-brain barrier (Sears et al., 1985), disrupts cerebral blood flow (Andersson et al., 1981), impedes excitatory amino acid neurotransmitter metabolism, e.g. glutamate and aspartate (Hindfelt et al., 1977). Efficient detoxification of ammonia requires coordinated effort between tissues to maintain ammonia homeostasis. Most catabolized amino acids are transaminated to form glutamate (Wright, 1995), and glutamate is metabolized by GLUD, GLS and GS (Hudson and Daniel, 1993). GLUD catalyzes the inter-conversion of glutamate to α-ketoglutarate and NH_4^+ (Labow et al., 2001). GS catalyzes the conversion of glutamate and ammonia into glutamine (Newsholme et al., 2003), and the reverse reaction is catalyzed by GLS (Curthoys et al., 1995). Glutamine therefore serves and functions as a major transport of ammonia in the body. The liver is the central site for N metabolism and N is transported from peripheral tissues, e.g. intestines, muscles and lungs) to the liver as glutamine (Haussinger, 1989).

In LRFI at day 28-35, there is down-regulation of GLS, GLUD1 and GS in the duodenum suggesting a possible down-regulation of the inter-conversion of glutamine, glutamine and keto-acids. The intestine may not be the prominent tissue for generating N for excretion in LRF

compared to HRFI. Mammalian skeletal muscles express high GS and low GLS (Wu et al., 1991) and this was also found in the chicken where high level of glutamine corresponded with increased muscle protein synthesis (Watford and Wu, 1995). GLS was up-regulated in LHRI at day 28-35, and GS was down-regulated but not significant implying that the rate of protein synthesis might be lower in LRFI compared to HRFI. The suggested decline in protein synthesis at day 28-35 in LRFI may be in concordance with its BWG which was reduced compared to HRFI. Also both GLS and GS were up-regulated possibly to maintain a balance between glutamine and glutamate. GLUD1 was down-regulated in the kidney in LRFI. It appears that at day 28-35 the physiology of LRFI supports reduced levels of glutamine entering the purine biosynthesis pathway compared to HRFI. The duodenum did not appear to play a major role in generating ammonia in LRFI at day 35-42 either. However, there was a down-regulation of GLS and up-regulation of GS in the breast muscle suggesting an increased in conversion of glutamate to glutamine in LRFI compared to HRFI. Since BWG between LRFI and HRFI were similar at day 35-42, glutamine produced in the muscle might be transported to the liver and kidney to enter into the purine biosynthesis pathway for *de novo* purine synthesis and/or uric acid for excretion.

The GS, GLUD1 and GLS expression differences between LRFI and HRFI at day 35-42 together with their corresponding FI and BWG suggest that there is a higher production of glutamine from breast muscle of LRFI than HRFI. Whereas LRFI at day 28-35 generates less glutamine in the liver and kidney, at day 35-42, the reverse seems plausible.

Glutamic-oxaloacetic transaminase (GOT) is a pyridoxal phosphate-dependent enzyme which exists in cytoplasmic and inner-membrane mitochondrial forms, GOT1 and GOT2, respectively. GOT plays a role in amino acid metabolism, and urea and tricarboxylic acid cycles

(Marvrides and Christen, 1978). GOT catalyzes the interconversion of aspartate and α-ketoglutarate to oxaloacetate and glutamate. During aspartate breakdown the glutamate formed undergoes deamination to form NH_4^+ which is excreted (Kirsch et al., 1984). Asparagine synthetase (ASNS) catalyzes the amidation of aspartate and glutamine in the presence of Mg^{2+} to asparagines. At day 28-35, ASNS was up-regulated in the duodenum, muscle and liver and down-regulated in the kidney of LRFI, while both GOT1 and GOT2 were down-regulated in all four tissues. The dynamics of ASNS, GOT1 and GOT2 at day 28-34 suggest increased glutamate formation and reduced conversion of glutamine to asparagine in the duodenum, muscle and liver, but in the kidney of LRFI, both ASNS and GOT1 were down-regulated. However, at day 35-42, besides GOT2 which was down-regulated in the duodenum, ASNS, GOT1 and GOT2 were all up-regulated in the breast, liver and kidney tissues in LRFI compared to HRFI. The putative amidation of aspartate in the muscle, liver and kidney could potentially reduce the level of glutamine entering the purine biosynthesis pathway for excretion and/or *de novo* purine synthesis.

Aldehyde dehydrogenase 4 family member A1 (ALDH4A1) is a mitochondrial matrix NAD-dependent dehydrogenase which catalyzes the conversion of pyrroline-5-carboxylate to glutamate in the proline degradation pathway (Valle et al., 1979). ALDH4 has been identified as a target of p53 and expression of ALDH4 mRNA was induced in response to DNA damage (Yoon et al., 2004). At day 28-35, ALDH4A1 was down-regulated in all four tissues, but was up-regulated in the muscle, liver and kidney at day 35-42 in LHRFI. NI in LRFI was significantly lower at both age periods, but at day 28-35, BWG declined whereas at day 35-42, BWG was similar compared to HRFI. It is likely that proline may be used as a N source by degradation into glutamate, especially at day 35-42 in order to maintain growth in LRFI. The CCBL1 gene

encodes for a cytosolic enzyme responsible for the metabolism of cysteine conjugates of certain halogenated alkenes and alkanes, and reactive metabolites (pyruvate, ammonium and a sulfur containing fragment) from such metabolism can lead to nephrotoxicity and neurotoxicity (Han et al., 2009). If the sulfur-containing fragment is reactive, the parent cysteine S-conjugate may be toxic, especially to kidney mitochondria (Cooper and Pinto, 2006). CCBL1 was down-regulated in the duodenum and up-regulated in the liver at day 28-35. At day 42, CCBL1 was up-regulated only in the duodenum of LRFI. CCBL1 did not appear to play any major role in the kidney of both LRFI and HRFI.

Glutamine phosphoribosylpyrophosphate amidotransferase (GPAT) is an enzyme encoded by the phosphoribosyl pyrophosphate amidotransferase (PPAT) gene, and GPAT is the first limiting enzyme in the purine biosynthesis pathway (Moat and Friedman, 1960; Koenigsknecht et al., 2007). It catalyzes the conversion 5-Phosphoribosylpyrophosphate (PRPP) to 5-Phosphoribosylamine (PRA) (Koenigsknecht et al., 2007) by deamination of glutamine. PRA through several steps yield inosin 5'-phosphate (IMP). IMP can be converted to adenosine 5'-monophosphate (AMP) and guanosine 5'-monophosphate (GMP) in order to synthesize the nucleic acids adenine and guanine. In uricotelic species including chickens, IMP is also converted to inosine and subsequently to uric acid. The final enzyme in the uric acid formation is xanthine dehydrogenase (XDH) (Remy and Westerfeld, 1951; Richert and Westerfeld, 1951), and in chickens Chou (1972) demonstrated that the kidney is the relatively important in the synthesis of uric acid than liver. At day 28-35, PPAT was down-regulated in the duodenum and kidney of LRFI suggesting a possible relatively lower deamination of glutamine compared to HRFI. Lower rate of deamination could lead to less N going through the rest of the purine cycle and could be the basis of less FN in LFRI. At day 35-42, there was a down-regulation of PPAT

in breast muscle of LRFI. It could be that glutamine is needed to be maintained for protein synthesis rather than entering the purine biosynthesis pathway for deamination. PPAT was up-regulated in the liver and kidney. Zhou et al. (1993) showed that PPAT is subject to feedback regulation by adenine and guanine nucleotides, and Yamoaka et al. (2001) also confirmed that the rate of *de novo* purine synthesis, DNA and protein synthesis rates and rate of cell growth exert feedback regulation on PPAT. The correlation between RFI and LRFI was positive indicating lower FN in LRFI compared to HFRI. Taken together, the up-regulation of PPAT in LRFI at 35-42 could be in response to the need to synthesize more purine nucleotides than to reduce ammonia burden, as FN was lower in LRFI than HRFI. Biosynthesis of purine nucleotides increases the growth rate mainly through ATP production and G(1)/S transition (Kondo et al., 2000). We can therefore hypothesize that chickens in the LRFI line despite significantly reducing their FI (protein intake) maintain their BW by excreting less nitrogen and putatively synthesizing more purines to maintain growth compared to the HRFI chickens. This is supported by Burns and Buttery (1984) who reported that the ratio of adenine to guanine nucleotides and the interaction between substrates and purine nucleotides are involved in the regulation of uric acid biosynthesis in chickens.

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Table 5.1 Means (±SE) of feed efficiency parameters in a chicken population divergently selected for low (LRFI) or high (HRFI) residual feed intake

Trait	Period	LRFI	HRFI	Pr>F
MBW	28	171.43±1.15	173.99± 1.27	0.1357
BWG	28-35	394.37±4.90	420.89± 5.42	0.0003
FI	28-35	707.89±8.65	803.15± 9.58	<0.0001
RFI	28-35	-31.86±6.38	32.33± 7.07	<0.0001
FCR	28-35	1.80±0.02	1.93± 0.02	<0.0001
MBW	35	222.20±1.47	227.81± 1.62	0.0103
BWG	35-42	376.39±4.72	372.09± 5.23	0.5406
FI	35-42	776.60±9.96	886.72±10.91	<0.0001
RFI	35-42	-41.35±8.33	54.80± 9.22	<0.0001
FCR	35-42	2.13±0.04	2.49± 0.05	<0.0001
ABFW	42	28.93±0.64	30.52±0.69	0.0943
ABFY (%)	42	2.60±0.08	2.81±0.09	0.0885

[1]MBW = Metabolic body weight (BW); BWG = BW gain; FI = feed intake; FCR = feed conversion ratio; RFI = residual feed intake; ABFW=abdominal fat weight; ABFY=abdominal fat yield

[2]All traits measured in (g) except for FCR which was (g/g);

[3]Age (d) or age range (d) that trait was measured

Table 5.2 Primer pairs used to analyzed gene expression by quantitative RT-PCR, and size of product

[1]Gene Symbol	GenBank Accession No.	Forward Primer	Reverse Primer	Size (bp)
CCBL1	XM_003642282.1	CCA TTC AGC TGC CAT TCA GCA CTT	TCA CAG CTT TCA GGA CTG TGG GAA	186
GLS	NM_001031248.1	AAA TCT GCT GTT TGC CGC CTA CAC	CGT GCA ATG CTG TTC GTG AGT CAT	110
GLUD1	XM_421497.3	ATC TGC AAC TGG TCG TGG TCT CTT	ATG CAA GCC CAC GTT ACC AAA TCC	139
GOT1	NM_205321.1	TCC CAG ATG GAG AAG ATT GTG CGT	AGA CCC GAT CTG CCA TTG TCT TCA	139
GOT2	NM_205523.1	ACG TGT AGC CTT GAC TTC ACT GGT	AGG AGG TTC CGT TTC TTC ACC ACA	161
GS	S45408.1	TAC ACA CAA AGC CAG CAG ACG AGA	CAG CGC TGG GCC TAA CAA TCA AAT	80
ASNS	NM_001030977.1	TGT CCA GTG TCT GAG TGC CAT GAA	AGC ACA GCC ACA GAT AAG GGA ACT	176
ALDH4A1	NM_001252104.1	GCG TTG GGT ACT TTG TTG AGC CAT	AAT TAT GCT CCT CTC CTG GGC GAA	200
PPAT	NM_001004401.1	ATA TTC GTG TGG CTT CAC CTC CCA	ACG ATA CGA GCC CTT CCA CAG AAA	162
β-actin	NM_205518.1	AGA CAT CAG GGT GTG ATG GTT GGT	TCC CAG TTG GTG ACA ATA CCG TGT	125

[1]The genes are: cystein conjugate-beta lyase 1 (CCBL1); glutaminase (GLS); glutamate dehydrogenase 1 (GLUD1); glutamic-oxaloacetate transaminase 1 (GOT1); glutamic-oxaloacetate transaminase 2 (GOT2); glutamine synthetase (GS); asparagine synthetase (ASNS); aldehyde dehydrogenase 4, member 1 (ALDH4A1); phosphoribosyl pyrophosphate amidotransferase (PPAT); and beta-actin (β-actin).

Table 5.3 mRNA expression of genes at day 28-35 in the Nitrogen recycling pathway in chickens divergently selected for low (LRFI) or high (HRFI) residual feed intake

		Fold change			
Symbol	Gene name	Duodenum	Muscle	Liver	Kidney
ALDH4A1	Aldehyde dehydrogenase 4, member 1	-1.34	-1.34	-1.01	-2.06
ASNS	Asparagine synthetase	1.58	1.32	1.80	-1.44
CCBL1	Cystein conjugate-beta lyase 1	-1.21	-1.16	1.39	-1.10
GLS	Glutaminase	-1.78	1.26	1.38	-1.10
GLUD1	Glutamate dehydrogenase 1	-1.63	-1.01	-1.05	-1.29
GOT1	Glutamic-oxaloacetate transaminase 1	-1.81	-1.17	-1.20	-1.36
GOT2	Glutamic-oxaloacetate transaminase 2	-1.45	-5.03	-1.12	-1.19
GS	Glutamine synthetase	-1.29	-1.14	1.85	1.05
PPAT	Phosphoribosyl pyrophosphate amidotransferase	-1.38	1.02	1.09	-1.29

Table 5.4 mRNA expression of genes at day 35-42 in the Nitrogen recycling pathway in chickens divergently selected for low (LRFI) or high (HRFI) residual feed intake

		Fold change			
Symbol	Gene name	Duodenum	Muscle	Liver	Kidney
ALDH4A1	Aldehyde dehydrogenase 4, member 1	1.13	1.36	1.83	1.97
ASNS	Asparagine synthetase	1.16	1.19	1.72	1.65
CCBL1	Cystein conjugate-beta lyase 1	1.42	1.15	-1.08	-1.02
GLS	Glutaminase	1.33	-1.26	-1.11	1.47
GLUD1	Glutamate dehydrogenase 1	1.12	1.13	1.43	1.23
GOT1	Glutamic-oxaloacetate transaminase 1	-1.03	1.19	1.20	1.34
GOT2	Glutamic-oxaloacetate transaminase 2	-1.30	1.22	1.36	1.22
GS	Glutamine synthetase	-1.02	3.11	2.25	1.62
PPAT	Phosphoribosyl pyrophosphate amidotransferase	1.14	-1.31	1.73	1.45

CHAPTER 6

General conclusion

Our understanding of genes that affect feed efficiency in chickens is inadquate. Explosion in molecular technology techniques has made it possible to delineate the molecular basis of feed efficiency. Genetic markers in the genes that affect feed efficiency can be developed to assist in conventional selection strategies. The general goal of this thesis is to study molecular and biological functions of genes that underlie feed efficiency and nitrogen recycling in a chicken population divergently selected for feed efficiency. We identified genes pathways associated with residual feed intake (RFI) through transcriptional profiling of duodenum at two different ages in a chicken population divergently selected for low (LRFI) or high (HRFI) RFI. The global gene expression differences in LRFI and HRFI were assessed by the Affymetrix GeneChip® Chicken Genome Array and real-time RT-PCR at days 35 and 42. The Ingenuity Pathway Analysis program was used to identify canonical and gene network pathways associated with RFI. Gene expression differences between LRFI and HRFI suggest that RFI can be explained by differences in (1) cell division, growth, proliferation and apoptosis, (2) protein synthesis, (3) lipid metabolism, and (4) molecular transport of cellular molecules. Chickens selected for improved RFI achieve efficiency by reducing feed intake with a nominal or no change in weight gain up-regulating CD36, PPARα, HMGCS2, GCG and down-regulating PCSK2, CALB1, SAT1 and SGK1. Chickens selected for reduced RFI via reduced feed intake with no change in weight gain achieve feed efficiency for growth by up-regulation of genes that reduces appetite with increased cellular oxidative stress, prolonged cell cycle, DNA damage and

apoptosis in addition to increased oxidation of dietary fat and efficient fatty acids transport from the intestines.

We also investigated the gene expression differences in the avian mechanistic target of rapamycin (mTOR). The mTOR is a highly conserved serine-threonine kinase that serves as intracellular energy and nutrient sensor and regulates cell division, growth, survival and apoptosis. The relationship of mTOR in mediating feed intake and growth in poultry is unknown. We studied genes in the mTOR pathway in duodenal and liver tissues at day 35 and 42 in LRFI and HRFI. In the duodenum mTOR was upregulated in the LRFI at both ages compared to the HRFI. Other genes differentially expressed at day 28-35 include AKT, EEF2, EIF4EBP1, PDK1, RPS6KP1, MLST8, GHRL, PI3K, FOXO1 and MDM2. At day 35-42, there was no change in expression of mTOR target RPS6KP1, and also MDM2. In the liver, changes in mTOR genes primarily occurred at day 42. There were also differences in plasma insulin and IGFI levels at day 28-35 between the LRFI and HRFI, but no such differences at day 35-42. In the duodenum at day 28-35, the PI3K/mTORC1 pathways are putatively activated with differential autophagy and apotosis in the LRFI line whereas at day 35-42, much of the regulation in the duodenum occurs through the AKT/mTORC2 pathways. In the liver, expression differences suggest mTORC1 control at day 35-42. We hypothesize that there could be nutrient deprivation in the duodenum cells of the LRFI line at day 28-35 resulting from active transport of amino acids from the duodenum rather than from starvation since all birds from both the low and high feed efficient lines were fed *ad-libitum* on the same diet.

We further studied the gene expression differences in the nitrogen recycling pathway to ascertain the molecular basis of nitrogen excretion in the chicken line divergently selected for feed efficiency at day 35 and 42 using duodenum, *Pectoralis major*, liver and kidney tissues. The

P major was the major source of glutamine in LRFI at day 28-35. Also in LRFI, ALDH4A1, CCBL1, GLS, GLUD1, GOT1, GOT2 and PPAT were down-regulated in all 4 tissues while ASNS and GLS were up-regulated in the muscle. At day 42, ALDH4A1, GOT2 and GS were up-regulated in the muscle of LRFI. The ALDH4A1, ASNS, GLUD1, GOT1, GOT2, GS and PPAT genes were up-regulated in LRFI compared to HRFI. There was a significant positive correlation between RFI and fecal N. Gene expression changes in GLS and GS at day 28-35 suggested a reduced rate of protein synthesis in LRFI, which may reflect the decline in weight gain compared in HRFI. However, at day 35-42, gene expression differences between the chicken lines suggested that the putative reduction in deamination and the up-regulation of PPAT in the liver and kidney of LRFI relative to HRFI may be due to feedback regulation in response to purine nucleotides that are needed to maintain weight gain.

The genes that are differentially expressed between LRFI and HRFI are functionally associated with residual feed consumption. Genetic markers can be developed in the genes to aid selection for genetic improvement.