AMINO ACID NUTRITION AND GHRELIN O-ACYLTRANSFERASE MRNA CHARACTERIZATION IN THE BROILER

by

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(Under the Direction of Adam Davis)

ABSTRACT

Determining digestible amino acid requirements for broilers is essential for further improving feed efficiency and optimizing muscle accretion as broiler strains continue to be selected for better performance. Several trials were conducted with Cobb 500 FF and Cobb 500 FF x Hubbard M99 broilers to determine the digestible lysine (dLys) and threonine (dThr) requirements at varying ages and grow-out conditions. In the U.S., the number of birds raised to 49 days has increased to meet the demands of the breast products produced by these sized birds. The dLys requirement calculated in two genetic crosses of broilers from 35 to 49 days of age in this research will allow poultry nutritionists to formulate diets that better meet the requirement for this essential nutrient, which is critical for optimal breast meat yield. There had been a trend by poultry nutritionists to increase dietary dThr levels based on several reports, which indicated that the increased levels of dLys used to maximize breast yield increased the dThr requirement. The current research indicated that increasing the dThr levels in diets fed from 14 to 28 days of age is not warranted. Although great strides have been made in optimizing broiler performance, continued improvement will result from a better understanding of the regulatory mechanisms involved in feed intake and energy metabolism. One hormone implicated in the regulation of

energy homeostasis and feeding behavior is ghrelin, which is activated through n-octaoylation carried out by the enzyme ghrelin O-acyltransferase (GOAT). Through real time RT-PCR, GOAT mRNA expression was detected in a few broiler tissues, but primarily expression was observed in the proventriculus. Furthermore, fasting caused up-regulation of GOAT mRNA expression in the proventriculus. By determining digestible amino acid requirements and better understanding the role of the ghrelin-GOAT axis in broilers, production efficiency can be optimized. Thus, fewer resources will be utilized for rearing the birds and less waste will be generated, thereby improving the environmental stewardship of the poultry industry while optimizing economic gains.

INDEX WORDS: broiler, broiler breeder, lysine, threonine, heat-stress

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DEDICATION

I dedicate this work to my amazing family. Without your unwavering and boundless support, this path would have been much more difficult. My gratitude to you is immeasurable for the countless hours spent consoling and encouraging me through my work in the midst of your chaotic lives. I love you and I am so proud of all your accomplishments during the last few years.

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW FOR BROILER AMINO ACID NUTRITION

General Overview

Though crude protein and essential amino acid research has been well documented in chickens for decades (Gordon and Maddy, 1956; Coon and Ngo, 1974; McNaughton and Reece, 1984; Sibbald and Wolynetz, 1986; NRC, 1994; Kidd et al., 1997; Kerr et al., 1999a; Corzo et al., 2006; Gonzalez-Esquerra and Leeson, 2006; Rostagno et al., 2007; Dozier et al., 2008; Baker, 2009; Dozier et al., 2010; Everett et al., 2010; Mehri et al., 2012), continuous genetic selection for better growth rate, feed conversion, and meat yield in broilers necessitates perpetual requirement analysis and adjustment of diet formulation. Relative to genetic stock from 1957, the modern broiler strain studied by Havenstein and colleagues, was nearly 5 times heavier and had a feed conversion ratio of approximately 1.46 whereas the 1957 broilers had feed conversion ratios of 2.34 for the same grow-out time period (Havenstein et al., 2003b). Due to the increased efficiency and 50 to 80 fold body weight increase at six to seven weeks of age, modern broilers need more nutrient and protein dense diets to achieve optimal growth (Havenstein et al., 2003; Kidd et al., 2004; Dozier et al., 2008). Today, broilers destined for the small bird market are grown to approximately 1.5 kg in five weeks (Corzo et al., 2005), whereas broilers raised for the further processed specialty markets are often grown between six to eight weeks of age in order to gain maximum breast meat. These heavy broilers will often reach a live market weight of 3 to 4

kg and require several stages of diets throughout the grow-out period (Corzo et al., 2002; Corzo et al., 2003; Dozier et al., 2008; Dozier et al., 2010).

Of the 20 known amino acids utilized by animals, chickens are unable to synthesize 10 in sufficient quantities to meet their biological needs and therefore must obtain them from their diet. These indispensable amino acids are: arginine, lysine, leucine, isoleucine, valine, threonine, tryptophan, phenylalanine, histidine, and methionine. The remaining 10 dispensable amino acids can be synthesized by the bird utilizing carbon and nitrogen substrates and are thus often referred to as non-essential. Amino acids, mainly in the form of protein, are present in various proportions in feed ingredients utilized for poultry diets, but some amino acids may also be supplemented in diets as synthetically manufactured crystalline powders or liquids. Currently, crystalline DL-methionine, L-lysine, L-threonine, L-tryptophan, and L-valine are commercially available as feed additives at economically feasible prices for the poultry industry.

Amino acids are incorporated into myofibers and are necessary for muscle accretion. For the broiler industry where maximal meat yield is the ultimate goal, dietary protein as the primary source of amino acids is inherently a central concern for nutritionists. Formulating diets deficient in one or more of the essential amino acids negatively affects performance and meat yield, whereas providing excess dietary amino acids increases feed cost, nitrogenous excretion, and potentially leads to amino acid interactions which can limit feed intake, influence amino acid transport and increase amino acid catabolism, all of which lessen bird performance.

Dietary Crude Protein

Dietary total nitrogen is derived from amino acids as well as non-protein compounds such as nucleic acids, flavonoids, terpenoids, benzoic, and cinnamonic acids. Consequently, nitrogen content analysis of diets is not directly correlated to the quantity or bioavailability of amino acids

of feedstuffs. Furthermore, a number of factors can affect protein quality including digestibility and indispensible amino acid concentration profiles. For example, the nutritional value of many crude protein sources decreases as a result of processing. In cottonseed and sesame seed meal production, cooking during oil extraction yields gossypol derivatives and reducing sugars that bind lysine and render this amino acid unavailable for absorption (Lyman et al., 1959). Similarly, in corn and soybean meal production, lipid oxidation of unsaturated fatty acids can involve the R groups of amino acids such as lysine, cysteine, methionine, and tryptophan thus reducing their bioavailability (Parsons et al., 1991). On the other hand, undercooking soybeans fails to denature the trypsin inhibitor naturally present in soybeans and consumption of soybean meal with active trypsin inhibitor adversely affects broiler growth performance (McNaughton et al., 1981; Parsons et al., 1991; Batal and Parsons, 2003).

The difficulty in formulating diets based on a crude protein level stems from the discrepancy between true amino acid digestibility analyses of feed ingredients versus high throughput analyses of amino acid content of feedstuffs which are utilized most often. The amino acid profiles of various ingredients can be determined relatively quickly via high performance liquid chromatography (HPLC) and ion exchange chromatography. However, these values are indicative of the total amino acid content and do not reflect actual digestibility and availability of the amino acids in the ingredient. For quantification of amino acid digestibility, cecectomized roosters are often utilized (as will be discussed shortly) to define the difference between the amino acid content of the ingested feed ingredient and the resulting feces. Theoretically, the difference reflects the amino acids that are absorbed and are available to be utilized for growth, production, and maintenance of the bird. A disadvantage to this technique, however, is higher cost and a longer timeframe for analysis. Subsequently, diet formulation

software programs contain nutrient digestibility values of feedstuffs based on previously determined experimental averages, which introduces error into formulation models. To avoid feeding inadequate amino acid levels, nutritionists create feed safety margins by adding additional protein, which increases the cost of the diet. By utilizing synthetically manufactured amino acids rather than crude protein, bioavailability discrepancies can be minimized but more importantly the total amount of protein fed can be reduced.

Diet Formulation on a Digestible Amino Acid Basis

Formulating poultry diets on a digestible amino acid basis accounts for individual amino acid bioavailability. Availability is dependent on the bird's ability to digest, absorb, and incorporate the amino acid as a building block for organ tissues or use a required metabolic end product derived from the amino acid. A number of methods have been developed to determine amino acid digestibility and/or availability through in vitro and in vivo assays measuring serum amino acid levels, growth performance, ileal or fecal content, enzymatic, chemical, and/or microbiological reactions (Ravindran et al., 1999a; Ravindran et al., 1999b; Adedokun et al., 2009). In this manner, various feedstuffs have been evaluated including cottonseed meal, spent-hen meal, meat and bone meal, corn and soybean meal. Utilizing these results, poultry nutritionists have been able to make better feed formulations that have decreased feed intake, improved feed to gain ratios, improved weight gain, and increased breast meat yield for market weight-reared broilers (Fernandez et al., 1995; Rostagno and Barbosa, 1995; Douglas and Parsons, 1999). Additionally, formulating on a digestible amino acid basis decreases the need for feed safety margins and thus lowers protein inclusion levels, which in turn has reduced nitrogenous excretion and overall feed cost (Summers, 1993; Nahm, 2007).

Utility of the Chick Growth Assays

Greater precision can be achieved by combining feedstuffs analysis methods. Despite the ability of chickens to be able to digest certain ingredients, nutrient interactions may decrease the bioavailability of the ingredient. The latter affect can be seen in amino acid diets that have highly digestible ingredients, but which result in poor growth performance due to amino acid imbalances. Hence, determining the digestibility of a feed ingredient through analysis with cecectomized roosters can then be combined with chick growth assays to measure the true bioavailability of the ingredient's content. In growth assays, provisioning chicks with diets variable in the test ingredient, allows nutrient interactions or imbalances to be detected if growth performance and or feed conversion is sub-optimal.

Another application of growth assays is to determine the appropriate inclusion levels of test amino acids required for optimal performance. For example, diets are formulated to be deficient in a test amino acid after which, the amino acid is supplemented at pre-determined levels. The result is a series of diets with incrementally titrated amino acid concentrations that allow growth performance to be directly attributed to the ingested amount of the amino acid. Graphically, performance response criteria such as body weight, body weight gain, carcass yield, and cut-up yield, all respond positively to increasing dietary amino acid levels. Broilers reared on the amino acid deficient diets have poor growth but as dietary amino acid levels increase, birds grow larger until a requirement plateau is reached. The law of diminishing returns can be applied to this relationship because higher amino acid supplementation increases feed cost but genetic and physiological constraints limit broiler growth even if dietary protein levels continue to rise.

In contrast, feed efficiency and lipid accretion are negatively correlated and decrease with higher amino acid levels (Grisoni et al., 1991; Han and Baker, 1994; Leclercq, 1998; Mack et al., 1999; Dozier et al., 2010). Birds fed denser amino acid diets can consume less to meet their protein requirement, thus improving feed to gain ratios, whereas broilers reared on marginally deficient diets must increase feed intake to obtain the same amount of the test amino acid to meet their growth requirements. Broilers fed amino acid sufficient diets, in other words protein sufficient diets, exhibit reduced carcass adiposity and abdominal fat pad weights (Cabel and Waldroup, 1991; Summers et al., 1992; Deschepper and De Groote, 1995; Sklan and Plavnik, 2002; Chendrimada et al., 2007) because more energy is required to sustain the increased muscle synthesis and maintain increased amounts of muscle tissue relative to fat tissue (Sibbald and Wolynetz, 1986). Furthermore, de novo fatty acid synthesis is decreased with higher levels of dietary protein in large part due to the suppression of malic enzyme activity, which produces the NADPH necessary for de novo fatty acid synthesis (Tanaka et al., 1983; Grisoni et al., 1991; Adams and Davis, 2001). Conversely, when a lack of amino acid building blocks limits muscle growth, dietary energy is not utilized for tissue accretion and the excess is stored in adipocytes.

Lowering Dietary Crude Protein

As summarized in the U.S. Livestock and Poultry Feed Use and Availability publication 70% of live production costs is feed, and the most expensive component of the feed is protein (Schnepf, 2011). As a result, for broilers in the U.S., it is customary to formulate least-cost diets where crude protein inclusion levels are minimized to increase the margin of profitability between feed expenditures and meat yield profits. Unfortunately, broilers reared on sub marginal dietary protein levels exhibit poor growth performance, lower muscle yield, and higher fat accumulation (Cabel and Waldroup, 1991; Grisoni et al., 1991; Deschepper and De Groote,

1995). Thus diets are balanced based on fluctuating market prices of raw feedstuffs as well as prices of specific meat components of the broiler carcass. Within the last decade, consumer demand for white meat has increased the market value of breast filets and tenders. As reviewed by Dozier et al. (2008), breast meat prices impact gross feeding margins more profoundly than the price of feed ingredients, thus, protein inclusion levels should be set accordingly after assessing both market factors.

Lowering dietary crude protein inclusion also has a bearing on the environmental impact of the poultry industry. Poultry manure contains nitrogen and phosphorus compounds excreted by the bird. In broiler production, litter and air quality are often a concern when excreted nitrogen compounds are converted to ammonia by bacteria in the litter. High levels of ammonia can cause poor growth performance and irritation of the conjunctiva in birds (al Homidan et al., 1998; Miles et al., 2006). On the other hand, poultry litter is a natural fertilizer that can be spread in fields to nourish plant crops. However, leaching of nitrogenous compounds into surface water can lead to eutrophication via nitrate and nitrogen contamination of water bodies while ammonia volatilization can acidify and negatively impact air quality (Cooke et al., 2011; Valadao et al., 2011). Thus, attempts to regulate nitrogen content in litter and the manner by which it is utilized for fertilization has been a growing concern (Summers, 1993; Chapman, 1996; Bolan et al., 2010). One of many methods utilized to reduce nitrogenous waste, as reviewed by Nahm (2007), is to formulate diets to exactly meet the amino acid requirements of birds based on the ideal protein concept. By providing the exact quantities of amino acids required by the birds, deficiencies and excesses can be avoided thereby simultaneously reducing feed cost and improving the industry's environmental stewardship.

Ideal Protein Concept

The dietary requirement for protein is a requirement for individual amino acids rather than for crude protein (NRC, 1994). In 1981, the Agricultural Research Council proposed the ideal protein concept, where the concentrations of indispensable amino acids are rationed relative to lysine (Heugten, 1999). Lysine was chosen as the reference amino acid 1) due to its importance in muscle accretion relatively to other amino acids, 2) because it is a significant limiting amino acid in corn-soybean based broiler diets and 3) because analyzing the amino acid requirement for lysine is relatively uncomplicated (Baker, 1997). Once the requirement for lysine is established, all other amino acid levels can be adjusted appropriately to the requirement of lysine (Han and Baker, 1994). The requirement for all amino acids can be defined as a function of body maintenance, growth, and production. In order to perform the latter functions, specific quantities and ratios of amino acids are necessary for the physiological reactions involved in tissue accretion and maintenance. Consequently, amino acid requirement patterns change with age and production status.

In theory, the use of crude protein can be eliminated if broiler diets are formulated to fulfill the exact amino acid requirement patterns of the birds through the use of synthetic amino acids. In 1985, Edmonds et al attempted to reduce crude protein inclusion levels from 24% crude protein to 16% crude protein while supplementing diets with limiting amino acids, however, synthetic amino acids failed to salvage poor performance. Subsequent years of research led to the discovery that glycine (Dean et al., 2006), added in concert with limiting essential amino acids (Fernandez et al., 1994), was necessary in producing diets with crude protein inclusion levels as low as 16%. Subsequently, broiler performance has been successfully

restored in a number of studies when synthetic sources of the limiting amino acids were used to supplement crude protein reduced diets as reviewed by Dozier et al. (2008) and Baker (2009).

Establishing concrete ideal amino acid profiles for broilers is difficult and ongoing as birds are continuously selected for superior performance. Moreover, genetic strain, flock age, sex, environmental conditions, diet ingredients, and nutrient interactions have been reported to modify amino acid requirements (Han and Baker, 1993; Corzo et al., 2003; Corzo et al., 2006; Garcia et al., 2006; Dozier et al., 2010) and necessitate perpetual research in order to optimize broiler performance and maximize industry profits. The first 6 consecutively limiting essential amino acids for broilers fed conventional corn-soy based diets have been determined as: methionine, lysine, threonine, arginine, isoleucine and valine with the last three being limited essentially equally (Fernandez et al., 1994; Emmert and Baker, 1997; Baker et al., 2002; Mehri et al., 2012).

Lysine

In the ideal protein concept, lysine is the reference amino acid where all other indispensable amino acids are rationed and expressed as a percentage relative to lysine (Han and Baker, 1994; Emmert and Baker, 1997; Baker et al., 2002; Baker, 2009). Of the 10 essential amino acids, lysine has the greatest impact on breast muscle development due to its high percentage (8.4%) of incorporation into muscle relative to the other amino acids (Munks et al., 1945). In general, diets containing low dietary protein limit muscle synthesis by reduction of myogenic gene transcription and myofibril size (Duclos et al., 1993; Rosebrough et al., 1996; Tesseraud et al., 2003; Duclos, 2005; Tesseraud et al., 2006). Limiting dietary lysine, in particular, more profoundly impacts breast muscle formation relatively to other essential amino acids due to its high incorporation into the fast-twitch fibers of the *pectoralis major* and *minor*

(Munks et al., 1945; Tesseraud et al., 1996). Tesseraud et al. (1996) demonstrated that dietary lysine is essential for breast muscle formation and that lysine deficiency in chicks leads to significant total white meat yield reduction. Sklan and Noy (2004) also confirmed that limiting the supply of dietary lysine led to limited protein synthesis in concert with higher catabolism of other amino acids.

The bioavailability of lysine depends on multiple factors. As previously noted, Maillard reactions occur during oil extraction and processing of soybean, cottonseed, and sesame seed meals. In these reactions, free epsilon groups of lysine and free carbonyl groups of reducing sugars react to form lysyl-sugar compounds that cannot be digested or absorbed in the broiler gastrointestinal tract (Hurrell and Carpenter, 1981; Parsons et al., 1992; Fernandez and Parsons, 1996). Additionally, as reviewed by Lerner (1984), absorption of ingested lysine at the intestinal brush border and basolateral membranes involves sodium-dependent and sodium-independent transport systems. To that end, Chen et al. (2005) reported that the apparent ileal digestibility of arginine and lysine can be influenced by changes in dietary salt inclusion levels. Once in the circulatory system, lysine can also interact and decrease arginine retention in kidneys as the two amino acids compete for reabsorption in the renal tubules (Austic and Scott, 1975). This antagonism is further exacerbated by the stimulatory action of lysine upon renal arginase activity, which degrades arginine to ornithine and urea (Austic and Scott, 1975). On the other hand, dietary availability of lysine has been shown to augment anabolism and catabolism of other amino acids in particular, incremental increases in dietary lysine result in lower catabolism of arginine and threonine and improved utilization to a point (Sklan and Noy, 2004). When taking all factors into account, the difficulty in determining appropriate ratios to lysine as well as accurate requirements for all individual amino acids is understandable.

Threonine

In corn-soy based broiler diets, threonine is the third limiting amino acid. The threonine requirement has been studied extensively in young chicks and broilers grown to market weight (Austic and Scott, 1975; Davis and Austic, 1982; Kidd et al., 1997; Kerr et al., 1999b; Mack et al., 1999; Baker et al., 2002). However, genetic strain, sex, age, environmental conditions, and nutrient interactions can affect threonine utilization in broilers (Kerr et al., 1999b; Sklan and Noy, 2004; Samadi and Liebert, 2006; Mehri et al., 2012). For example, feeding chicks diets with threonine imbalances has been shown to increase hepatic threonine dehydrogenase activity (Davis and Austic, 1982; Davis and Austic, 1994; Davis and Austic, 1997). As summarized by Bird and Nunn (1983), threonine dehydrogenase is the primary threonine-catabolizing enzyme that converts L-threonine to 2-amino-3-oxobutyrate. In various species, threonine dehydrogenase activity has been reported to be up-regulated by increased dietary levels of other amino acids such as glutamic acid and leucine (Ernsting et al., 1992; le Floc'h et al., 1994; Davis and Austic, 1997; Weng et al., 1997).

Interactions between lysine and threonine have also been reported to affect breast muscle development. In separate trials Kidd et al. (1997) and Kerr et al. (1999b) demonstrated that dietary threonine requirements to maximize white meat yield increased as dietary lysine supplementation was increased to maximize breast yield. On the other hand, Everett et al. (2010) did not observe a significant lysine x threonine interaction for processing parameters, although interactions were noted for body weight, body weight gain, feed intake, and feed efficiency between these two amino acids. For young broilers, interactions between the latter amino acids may not be evident as the birds are not rapidly synthesizing breast muscle (Kerr et al., 1999b; Everett et al., 2010).

Threonine is highly incorporated into mucins produced by intestinal epithelial cells and is important for maintenance of intestinal integrity (Bertolo et al., 1998). Mucins are glycoproteins that contain high amounts of proline, threonine, and serine in their peptide backbones and are the major components of the intestinal mucosal barrier responsible for protecting the gastrointestinal tract (Roberton et al., 1991; Van Klinken et al., 1995). Faure et al. (2005) demonstrated that mucin synthesis is impaired in rat intestines when dietary threonine is restricted. Inflammation due to physical damage and/or gastro-enteric pathogens increases mucin synthesis and increases the requirement for threonine (Faure et al., 2007). Furthermore, raising the dietary threonine:lysine ratio during challenges with enteric pathogens has been shown to improve growth performance in broilers in relation to pair-fed, noninfected birds (Star et al., 2012). Thus, subclinical challenges by pathogens originating from top-dressed, previously used litter may influence digestible threonine requirements in broilers.

Heat Stress and Amino Acid Nutrition

Most broiler production in the United States occurs in regions where humidity is high and diurnal summer temperatures cause ambient temperatures in broiler houses to increase beyond the thermo-neutral zone of modern broiler strains. Erratic and/or prolonged exposure to such conditions can result in poor growth performance due to lower feed intake, altered muscle protein deposition, higher mortality, and immunosuppression of broilers (Bottje and Harrison, 1985; Young, 1990; Yahav et al., 1995; Temim et al., 2000b). Recognizing the effects of environment upon production, nutritionists attempt to reduce such effects by altering nutrient density with varying results (Temim et al., 2000a).

Rearing broilers under elevated environmental temperatures induces physiological and metabolic response that allow birds to cope with heat stress, but which, detrimentally impact

growth performance parameters (Yahav et al., 1995; Temim et al., 2000a; Temim et al., 2000b; Tankson et al., 2001; Dridi et al., 2008). Most often, high ambient temperatures fluctuate with diurnal cycles where morning and night temperatures are typically cooler. Relative to broilers confronted with constant heat-stress, birds reared under cyclic heat-stress conditions adapt and achieve superior growth (Deaton et al., 1984) by feeding most actively early and late in the day, thereby reducing metabolic heat expenditure during hours of maximum heat stress. To the contrary, persistent heat-stress conditions exacerbated feed intake suppression as the broilers minimize consumption throughout the light period and subsequently grow more slowly and inefficiently.

The amino acid requirements of broilers remain relatively similar between birds reared under elevated environmental temperatures versus broilers fed in ideal, thermo-neutral conditions (Han and Baker, 1993). However, for heat stressed birds, smaller body size and proportionately lower maintenance energy costs equate to lower feed intake and feed conversion ratios for genetic strain and age-matched broilers (Han and Baker, 1993). Modifying diets to increase amino acid density has been shown to alleviate the effects of low feed intake by increasing the broilers' net consumption of amino acids and thereby improving performance during heat stress (McNaughton and Reece, 1984; Corzo et al., 2003; Gonzalez-Esquerra and Leeson, 2006).

Analysis Methods for Nutritional Response Data

Amino acid requirements are often estimated through quadratic and broken-line regression analyses. Quadratic polynomial models are based on least squares analysis methods fitted to input (ie. test amino acid consumed) and output (ie. growth performance) data. These models clearly depict nutrient toxicities and "diminishing returns" effects when excessive

nutrient levels are consumed and output data remain relatively constant or begin to decline. On the other hand, quadratic models are thought to overestimate requirement levels because the requirement is determined at the vertex where the output is maximized rather than where a theoretical "break-point" is reached once the physiological threshold is met.

Broken-line models are commonly utilized to calculate the point at which output data first begin to plateau. The latter models contain an ascending line where, for example, growth performance linearly improves as dietary test amino acid levels are incrementally increased until the requirement is reached. Thereafter, output data points are relatively similar as the birds reach a physiological growth limit and cannot respond to increasing inclusion levels. This plateau segment has a slope equal to 0 and the intersection between the two lines is established as the requirement.

A hybrid between the quadratic and broken-line models is the broken-line quadratic model, which is believed to more accurately represent biological responses. Nutritional output data tend to exhibit slightly curved ascending segments until the requirement plateau is reached. Once the output data plateaus, the quadratic slope is once again set to zero and the requirement is calculated as the lowest input level that provides maximum output response.

Amino acid requirement calculations are typically based on software developed by Dr. Gene Pesti and colleagues, which can be accessed through http://www.caes.uga.edu/Publications/pubDetail.cfm?pk_id=7919&pg=np&ct=RB%20440&kt=&kid=&pid= (Vedenov and Pesti, 2007). Growth and yield data are subjected to both quadratic and broken-line regression analyses.

Summary

The dietary protein requirement for poultry is a requirement for individual amino acids. Amino acids are utilized in various physiological reactions and are an essential component of protein synthesis and myogenesis. Since the focus of commercial broiler production is to improve feed efficiency while increasing meat yield, dietary protein inclusion is understandably an important aspect of broiler nutrition research. Moreover, crude protein feedstuffs are the most expensive ingredients in poultry feed, thus reducing crude protein levels in broiler diets can potentially improve the industry's profitability. In addition, the amino acids associated with excess dietary protein are catabolized and the nitrogen component of the excess amino acids is excreted. Subsequently, reducing the amount of dietary protein also can decrease ammonia production in broiler facilities and reduce the environmental impact of poultry litter. Unfortunately, formulating diets even just marginally deficient in one of the essential amino acids results in depressed growth, feed efficiency, and lower meat yield. Failing to optimize growth of certain muscle groups, such as the breast muscles, has been shown to impact profitability of U.S. broiler producers more profoundly than feed ingredient prices. To lower dietary crude protein inclusion without compromising meat yield, several amino acids are currently synthetically manufactured in purified form and are available as feed supplements at prices reasonable for commercial use. However, determining the levels at which the amino acids should be rationed in feed has been an ongoing research focus for decades due to the difficulty in resolving the requirements of the various meat-yielding strains reared under various environmental conditions.

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CHAPTER 2

INTRODUCTION AND LITERATURE REVIEW OF GHRELIN AND GHRELIN O-ACYLTRANSFERASE

The Mammalian Ghrelin-GOAT System

General Overview

Ghrelin is a 28-amino acid peptide hormone involved in the regulation of energy homeostasis and growth hormone (GH) secretion. In 1999, Kojima et al., isolated ghrelin from rat gastric tissue and identified it as the endogenous ligand for the growth hormone secretagogue receptor (GHSR). Currently, there are two identified isoforms of GHSR, 1a and 1b, however only GHSR1a activation results in GH secretion in the pituitary (Howard et al., 1996). Ghrelin also has two identified forms; des-acyl ghrelin and acylated ghrelin both of which are found in circulation. Within this thesis, DAG will denote des-acyl ghrelin whereas ghrelin will indicate acylated ghrelin. The latter acylated form has a characteristic n-octanoic acid functional group on Ser3 (Thr3 in frogs), which is added to DAG by a reaction catalyzed by the enzyme ghrelin O-acyltransferase (GOAT). In 2008, two separate groups identified GOAT as the membrane bound O-acyltransferase (MBOAT4) responsible for modifying ghrelin. Currently, ghrelin is the only known peptide hormone with a fatty acid modification that is crucial for biological function as only the acylated form of ghrelin binds to and activates GHSR1 (Gutierrez et al., 2008; Yang et al., 2008a). Initially, the acylated form of ghrelin was believed to be the only biologically

active form, however, recent studies indicate that DAG is also involved in cell signaling pathways independent of the GHSR system (Inoue et al., 2010).

Ghrelin Gene Expression and Posttranslational Processing

In mammals, approximately two thirds of plasma total ghrelin (DAG + ghrelin) is produced by the stomach by cells within the gastric fundus mucosa while the remaining source of plasma total ghrelin is the small intestine (Date et al., 2000; Ariyasu et al., 2001; Gualillo et al., 2003). Other tissues that express ghrelin mRNA include the pancreas, heart, adipose tissue, adrenals, thyroid, pituitary, hypothalamus, placenta, ovary, and testes (Gnanapavan et al., 2002; Volante et al., 2002; Gronberg et al., 2008), but the mRNA expression levels in these tissues are much lower than what is expressed by the digestive tract (Gnanapavan et al., 2002). Of the total plasma ghrelin content, the concentration of DAG is approximately 50 times higher than that of ghrelin (Hosoda et al., 2000, Murakami et al., 2002).

In humans, the ghrelin (*GHRL)* gene encodes for a 117-residue peptide, preproghrelin. Preproghrelin undergoes proteolysis of the signal sequence (amino acids 1-23) to generate a 94 amino acid peptide known as proghrelin (amino acids 24-117). While this immature peptide form of ghrelin is still within the leaflets of the endoplasmic reticulum (ER), it may be subjected to acylation by GOAT (Yang et al., 2008b). Thus, two forms of proghrelin may be transported from the ER to the golgi body for further processing: unacylated proghrelin and acylated proghrelin (Kojima and Kangawa, 2010). Once within the golgi body, prohormone convertases (PC1/3) cleaves proghrelin after amino acid 51 (Zhu et al., 2006). The optimal pH for GOAT activity is between pH 7 and 8 while the optimal pH for PC1/3 activity is slightly acidic, between pH 5 – 6 (Zhu et al., 2006; Ohgusu et al., 2009). Hence, GOAT is likely located within the neutral environment of the ER while PC1/3 is located in the more acidic secretary granules of the

golgi body. Due to this partitioning, it is assumed that GOAT acylates preproghrelin and proghrelin prior to PC1/3 processing and cleavage (Zhu et al., 2006; Kojima and Kangawa, 2010). The result is the 28 amino acid ghrelin (amino acids 24-51) and a 66 amino acid C-terminal propeptide named C-ghrelin (amino acids 52-117) (Hosoda et al., 2003). Within C-ghrelin is the 23 amino acid hormone, obestatin (amino acids 76-98), which can be freed by further proteolytic processing of C-ghrelin (Lim et al., 2010; Romero et al., 2010). Collectively, this biosynthesis process generates several distinct peptides: ghrelin (acylated and des-acylated), C-ghrelin, and Obestatin.

While GOAT is the only enzyme capable of acylating ghrelin, PC1/3 is not the only prohormone converase capable of cleaving proghrelin. Depending on cell type, immunohistochemical colocalization studies also point to PC2 and furin as convertases capable of processing proghrelin. In gastric tissue, PC1/3 seems to be the exclusive convertase while in pancreatic cells PC2 is also available (Walia et al., 2009). Ablation of PC1/3 in mice, leads to loss of gastric proghrelin processing and therefore much lower levels of total circulating ghrelin. However, low levels of processed ghrelin are still detectable in the serum of knockout mice. Thus, non-gastric tissues are believed to be the source of serum acylated ghrelin by processing proghrelin with convertases other than PC1/3 (Zhu et al., 2006; Kojima and Kangawa, 2010).

Ghrelin Gene Locus Variants

Several transcripts of the *GHRL* gene locus have been identified. These forms differ either in the number of amino acids in the ghrelin peptide backbone or in the number of carbons of the acyl moiety attached to Ser3 by GOAT. As reviewed by Seim et al. (2009), there is increasing evidence that the preproghrelin gene is complex and can produce a number of RNA transcripts and peptides due to both alternative exon splice sites as well as alternative post-

translational cleavage sites. Studies focused on the human *GHRL* locus have resulted in the putative identification of upstream exons -1, 0, and extended exon 1 in addition to the originally discovered preproghrelin encoding exons 1, 2, 3, and 4 (Kanamoto et al., 2004; Nakai et al., 2004; Seim et al., 2007). Homologues to these upstream human exons have been identified in rodents and these variants are suspected to differentially control gene transcription and metabolic signaling pathways resulting in differential responses to ghrelin between species as well as tissues within an individual (Tanaka et al., 2001a; Tanaka et al., 2001b).

Of the putative transcripts, only two splice variant peptides have been isolated experimentally: des-Gln14-ghrelin and Δ3 C-ghrelin (Hosoda et al., 2000b; Hosoda et al., 2003; Jeffery et al., 2005a; Yeh et al., 2005). In rats and mice, the codon CAG (Gln14) is used as an alternative splicing signal that produces two different ghrelin mRNAs. One is translated into a full ghrelin peptide and the other encodes a des-Gln14-ghrelin precursor. Although des-Gln14-ghrelin is synthesized at lower levels relative to the 28 amino acid ghrelin, both forms are modified with n-octanoic acid, both bind GHSR, and both seem to exert similar biological activity (Hosoda et al., 2000b). On the other hand, the Δ3 C-ghrelin splice variant is the result of exon 3-deleted preproghrelin undergoing enzymatic cleavage to produce mature ghrelin and a 5' shortened C-terminal peptide, Δ3 C-ghrelin. The Δ3 C-ghrelin peptide is further cleaved and gives rise to an even shorter amino acid peptide (Δ3D). These peptides have been detected in several mouse tissues and may be involved in the control of cellular metabolic pathways because Δ3D is reportedly upregulated in human prostate and breast cancer cells (Jeffery et al., 2005b; Yeh et al., 2005). Since studies conducted to further understand the ghrelin metabolic axis often result in juxtaposing data, in the future, many of these differences may be contributed to tissue-

specific synthesis of the various peptides derived from the *GHRL* locus and the extent to which their ratios are manipulated within the cell by external factors.

Evidence supporting the hypothesis that peptide variants derived from the ghrelin gene differentially effect metabolic signaling pathways can be seen in the manner by which GHRL gene transcription is regulated. The existence of alternative promoters and upstream exon variants of preproghrelin exon 1 indicates that ghrelin may be regulated in several ways. First, the variation in length of 5' untranslated regions (5'UTRs) suggests that alternative 5' exons may be used to regulate translational efficiency by altering ribosomal attachment, namely leaky scanning and ribosomal re-initialization (Kozak, 2005; Hughes, 2006). Second, additional modes of gene regulation may stem from naturally synthesized natural antisense transcripts (NATs) derived from the human ghrelin antisense gene, *GHRLOS* (Seim et al., 2009). Reportedly, NATs are differentially expressed in gastric tissues, where they are found in low concentrations, versus the brain, testes, and the hypothalamus, where they are highly expressed. In the stomach, ghrelin and C-ghrelin production may be high due to the lack of NAT inhibition, whereas, in the brain and reproductive tissues these NATs may act as *cis* elements to fine-tune and suppress the ghrelin gene locus. Finally, NATs may also be interacting with other areas of the genome to act as *trans* elements, thereby regulating genes beyond the ghrelin locus (Seim et al., 2009; Seim et al., 2011).

Acyl ghrelin function

Ghrelin was originally discovered as the natural ligand for a growth hormone secretagogue receptor within the pituitary. Therefore, it is not surprising that ghrelin causes the potent release of growth hormone in somatotrophs both in vitro and in vivo in a dose dependant manner (Kojima et al., 1999; Peino et al., 2000; Hataya et al., 2001). The most striking

physiological effects of ghrelin, independent of GH releasing activity, is the stimulation of appetite and feeding behavior. Infusion of ghrelin, either intracerebroventricularly or peripherally, into mice or rats stimulates feeding behavior and if the injections are given long enough the animals will gain weight (Tschop et al., 2000; Wren et al., 2000; Kamegai et al., 2001). Ghrelin's stimulation of appetite is mediated by the neurotransmitters neuropeptide Y (NPY) and agouti related protein (AGRP) in the hypothalamic acurate nucleus region of the brain (Chen et al., 2004). However, feed intake in mice is not completely dependent upon ghrelin production because in ghrelin knockout mice the absence of ghrelin does not impair either growth or appetite (Sun et al., 2003).

Injection of ghrelin elevates plasma glucose levels in humans and mice (Broglio et al., 2003; Salehi et al., 2004). In addition, Gauna et al. (2005), found that porcine hepatocytes cultured with ghrelin released more glucose in both a time and dose dependant manner than untreated control cells. Furthermore, intraperitoneal injection of a GHSR antagonist will decrease fasting glucose levels in mice (Dezaki et al., 2004). In both humans and rats, ghrelin has been reported to decrease (Broglio et al., 2001; Egido et al., 2002; Broglio et al., 2003) or increase (Adeghate and Ponery, 2002; Date et al., 2002; Lee et al., 2002) plasma insulin concentrations. Since ghrelin increases plasma glucose levels, the rise in plasma glucose concentrations may be enough to stimulate insulin production. This indirect influence on insulin production by ghrelin may account for some of the conflicting reports on how ghrelin impacts insulin production. Studies concerning glucagon are limited, but Salehi et al. (2004) reported that ghrelin strongly stimulates glucagon release in murine islet cells in vitro. However, the same results have yet to be duplicated in vivo (Egido et al., 2002; Salehi et al., 2004).

In 2000, Tschop et al. reported that ghrelin not only indirectly effected adiposity by acting as an orexigenic peptide, but also reduced lipolysis in mice and rats. Chronic intracerebroventricular administration of ghrelin also regulates adipocyte metabolism by increasing glucose and triglyceride uptake as well as lipogenesis in white adipose cells in rats (Theander-Carrillo et al., 2006). Choi et al. (2003) also reported that rat adipocytes cultured with ghrelin had increased fat synthesizing capabilities, but Zhang et al. (2004) reported that adipocytes from a cell line overexpressing ghrelin had decreased rates of adipogensis compared to cells with typical ghrelin expression. However, both research groups agreed that ghrelin increased the rate of preadipocytes differentiating into adipocytes.

Leptin is produced by adipocytes in response to a positive energy balance and there is a negative correlation between plasma levels of total ghrelin and leptin (Otto et al., 2001; Tschop et al., 2001; Tolle et al., 2003). Interestingly, in cultured rat adipocytes ghrelin increases leptin secretion and the addition of a ghrelin antagonist will decrease leptin secretion (Giovambattista et al., 2006). Regardless of the effects of ghrelin upon leptin secretion in cultured adipocytes, it has been well documented that in vivo, ghrelin and leptin antagonize each other within the arcuate nucleus by modulating NPY mRNA expression levels and thereby feeding behavior (Shintani et al., 2001; Traebert et al., 2002; Kohno et al., 2008).

Des-Acyl Ghrelin

DAG is synthesized at approximately 50 fold higher levels than ghrelin and is therefore far more abundant in visceral tissues and in blood (Hosoda et al., 2000a; Murakami et al., 2002). This form of ghrelin does not bind GHSR because it lacks the GOAT appended moiety on Ser 3 required for biological activation of the receptor. Indeed, at physiological concentrations, radiolabled DAG does not substitute GHSR-bound ghrelin in the hypothalamus and pituitary and

does not result in GHSR-mediated GH release from the pituitary (Hosoda et al., 2000a). Due to these data, it was initially theorized that DAG was devoid of activity, however, more recent studies indicate that DAG is involved in a wide range of regulatory pathways including insulin homeostasis, orixigenesis, cell proliferation, and adipogenesis (Cassoni et al., 2004; Muccioli et al., 2004; Thompson et al., 2004; Asakawa et al., 2005; Granata et al., 2006; Sato et al., 2006; Granata et al., 2007). It has now become generally accepted that DAG is not a biologically inert hormone (Baldanzi et al., 2002; Murata et al., 2002; Gauna et al., 2005; Toshinai et al., 2006; Barazzoni et al., 2007; Zhang et al., 2008), but the mechanisms by which it causes its effects are unknown.

Obestatin

The name obestatin derives from the Latin roots "obedere", to eat, and "statin", to stop, because the original study conducted by Zhang et al. (2005) pointed to a potential anorexigenic effect of obestatin in rats. The researchers believed that obestatin activated GPR39 (G protein-coupled receptor 39) to exert the hunger repressing affects. Because both the orexigenic ghrelin and anorexigenic obestatin are produced from the same peptide, yet, have opposing actions, Zhang and colleagues suggested that no apparent ghrelin knockout phenotypes had been observed due to simultaneous elimination of both hormones.

An extensive body of research on obestatin has been generated over the last 7 years due to interest in using the hormone as a potential therapeutic agent against obesity. However, to date, most studies have repudiated the observations by Zhang et al. (2005) regarding the anorexic effects of obestatin and its ability to bind GPR39 (Lauwers et al., 2006; Bassil et al., 2007; Chartrel et al., 2007; Gourcerol et al., 2007; Nogueiras et al., 2007; Zizzari et al., 2007). Additionally, obestatin is reported to have a relatively short half-life in blood serum and, more

importantly, seems to be unable to permeate the blood-brain-barrier, which is needed to facilitate endocrine signaling between the CNS and peripheral tissues (Pan et al., 2006). Lastly, obestatin may not be present in blood circulation at all (Holst et al., 2004; Lauwers et al., 2006; Bang et al., 2007; Holst et al., 2007; Yasuda et al., 2007), but rather act as a short-distance signaling peptide with modifying roles in anxiety (Carlini et al., 2007), sleep (Szentirmai and Krueger, 2006), water homeostasis (Samson et al., 2007; Samson et al., 2008), cell proliferation (Camina et al., 2007; Pazos et al., 2007; Meszarosova et al., 2008), and apoptosis (Granata et al., 2007).

C-ghrelin

The 66 amino acid peptide C-ghrelin is transcribed from proghelin exons 2, 3, and 4. Several studies have detected C-ghrelin and other C-terminal peptides of ghrelin in mouse, rat, and human blood circulation (Pemberton et al., 2003; Bang et al., 2007). In fact, extensive size-exclusion HPLC, reverse-phase HPLC, and radioimmunoassay (RIA) data published by Bang et al (2007), suggest that not only does C-ghrelin circulate at higher concentrations than ghrelin, but also that obestatin is not present in serum. Moreover, these authors report that C-ghrelin tissue distribution correlates with ghrelin distribution and shows the same inhibition profile similarly to ghrelin following glucose ingestion and glucagon injections (Bang et al., 2007). Thus, given the high concentrations of C-ghrelin Seim et al. (2009) hypothesized that C-ghrelin may be responsible for the endocrine effects attributed to obestatin, whereas obestatin may be functioning more on an autocrine and paracrine level.

Ghrelin Receptor:

Discovery of GHSR preceded the successful isolation of its natural ligand, ghrelin (Howard et al., 1996). Because binding of synthetic substrates to the G-coupled protein receptor stimulated the release of growth hormone (GH), the receptor was reasonably named Growth

Hormone Secretagauge Receptor. Currently, there are two identified isoforms, GHSR1a and GHSR1b; however, only GHSR1a is activated by ghrelin and can initiate downstream signal transduction of secondary messengers to stimulate GH secretion. High affinity between ghrelin and GHSR1a is dependent on the presence of, at minimum, the first 5 N-terminal amino acids of ghrelin in conjunction with an acylated fatty acid moiety on the third N-terminal serine (Howard et al., 1996; Kojima et al., 1999; Matsumoto et al., 2001). The protein structure of GHSR1a is predicted to consist of an extracellular N-terminal domain, 7 transmembrane domains, and an intracellular C-terminal domain (Howard et al. 1996). GHSR1b is composed of 289 amino acids and lacks the first 77 amino acids encoded by the beginning of the second exon. It is predicted that this truncated form of GHSR has only the first 5 of the 7 transmembrane domains. Though the latter GHSR form cannot bind ghrelin, it may serve to downregulate availability of GHSR1a for binding.

The highest expression levels of GHSR1a mRNA are found in somatotrophs, cells responsible for GH secretion within the pituitary, and in the hypothalamus in the arcuate nucleus, an area crucial for neuroendocrine regulation of appetite stimulation (Guan et al., 1997; Kojima and Kangawa, 2005). However, GHSR1a mRNA is expressed at lower levels in a variety of other tissues such as heart, lung, liver, pancreas, intestine, adipocytes, thyroid, spleen, adrenal, ovarian and testicular tissue (Guan et al., 1997; Kojima et al., 2001; Gnanapavan et al., 2002; Barreiro et al., 2003; Gaytan et al., 2003). GHSR1b mRNA has been detected within the same tissues in which GSHR1a has been identified (Gnanapavan et al., 2002) but the mRNA expression of GHSR1b tends to be less than the expression of GHSR1a (Korbonits et al., 2001; Gauna et al., 2005).

Ghrelin O-acyltransferase (GOAT)

In early 2008, two independent research groups published the identification of ghrelin O-acyltransferase, the enzyme responsible for acylating ghrelin (Gutierrez et al., 2008; Yang et al., 2008a). Both groups speculated that the enzyme responsible for ghrelin acylation was a member of the Membrane Bound O-acyltransferase (MBOAT) family. Earlier work conducted on the *Drosophila wingless* and *porcupine* genes resulted in the discovery that Wingless activity was dependent on the action of the enzyme Porcupine (Kadowaki et al., 1996). Structural analysis of Porcupine led to the conclusion that the enzyme contained highly conserved amino acid sequences, which were also seen in other membrane-bound hydrophobic enzymes responsible for transfers of long-chain fatty acids (Hofmann, 2000) cholesterol moieties, and hydroxyl groups (Gualillo et al., 2008). Thus, Hofmann named the family of enzymes membrane-bound O-acyltransferases or MBOATs. Subsequent studies with the mammalian homolog to Wingless, named Wnt or Wnt-3a in rodents, confirmed that Porcupine is required for Wnt-3a Ser209 acylation, which in turn is required for Wnt-3a extracellular export from the ER (Takada et al., 2006). Since ghrelin and Wnt-3a are the only proteins known to have acylated serine residues, both Yang et al. (2008a) and Gutierrez et al. (2008) focused on the MBOAT family in their studies as primary candidates for ghrelin-acylating enzymes.

Gutierrez et al. (2008) selected twelve MBOAT sequences for gene-silencing. A cell culture system was developed with human medullary thyroid carcinoma cells (TT cell line) in conjunction with the MBOAT siRNA gene-silencing assays. One of twelve previously selected MBOAT genes was identified as the enzyme responsible for acylating ghrelin. To further confirm the findings, the candidate MBOAT4 gene was then co-transfected with proghrelin into human embryonic kidney (HEK-293) cells, which do not express either gene endogenously. The

resulting HEK-293 cell media was also subjected to mass spectroscopy (MS) analysis where DAG and Ser3-octanoyated ghrelin were detected. Moreover, the group demonstrated that GOAT catalytic properties are so highly conserved across species that zebrafish GOAT (~60% homologous to human GOAT) co-expressed with human ghrelin was still capable of acylating human ghrelin. Finally, the researchers generated GOAT knockout mice and determined that GOAT silencing abolished acyl ghrelin synthesis. The results all indicated MBOAT4 was the enzyme required for ghrelin acylation, which the researchers renamed ghrelin O-acyltransferase, or GOAT.

Simultaneously, but independently to Gutierrez et al. (2008), Yang and colleagues (2008a) determined that MBOAT4 was the orphan enzyme responsible for ghrelin acylation, which they also named GOAT. Their approach was based on an initial scan of the mouse genome to identify the sixteen members of the MBOAT family reported by Hofmann (2000). Primers were designed based on the sequences available in the National Center for Biotechnology Information (NCBI) database and the resulting cDNAs were transfected into the MIN-6 murine endocrine cell line which were capable of producing mature acylated ghrelin when cotransfected with MBOAT4 and mouse proghrelin jointly. To validate that MBOAT4 was responsible for ghrelin acylation and to test critical amino acids for GOAT activity, radiolabeled [^{3}H] octanoate and site-directed mutagenesis were combined. The series of studies conducted by Yang et al. (2008a) led to the conclusions that 1) GOAT is highly conserved across vertebrate species, 2) it is located in the ER where 3) it octanoylates proghrelin at Ser3 prior to transfer to the Golgi body, and 4) GOAT mRNA expression is highest in the stomach but the number of GOAT transcripts present are much lower than the number of ghrelin transcripts.

GOAT Expression Profile

In mammalian species, the mRNA for the enzyme has been detected in tissue from ovary, fallopian tube, placenta, breast, testes, prostate, fat, esophagus, stomach, duodenum, jejunum, ileum, ascending colon, descending colon, adrenal gland, spleen, liver, gallbladder, muscle, pancreas, kidney, myocardium, esophagus, lymphocytes, thyroid, lymph nodes, hypothalamus, and pituitary (Gutierrez et al., 2008; Yang et al., 2008a; Lim et al., 2011a). Highest mRNA transcript expression has been detected consistently in the stomach though GOAT mRNA is expressed at much lower levels when compared to ghrelin (Gutierrez et al., 2008; Yang et al., 2008a).

GOAT Substrate Specificity- CoA-conjugated Acyl Acid Binding Pocket

The most common forms of ghrelin are DAG and n-octanoyl modified ghrelin (Date et al., 2000; Hosoda et al., 2000b; Hosoda et al., 2003). In humans, the acyl carbohydrate chain length may vary from relatively short, C2:0 acetic acid, to relatively long, C16:0 tetradecanoic acid. However, all acyl acids must be conjugated with CoA because neither free acyl acids nor mixtures of CoA with free acyl acids result in acylated ghrelin (Gutierrez et al., 2008; Ohgusu et al., 2009). Despite the broad substrate specificity seen with human GOAT, the main form of gastric ghrelin is esterified with n-octanoic acid. Other acyl-modified forms do exist but are detected at much lower levels (Kojima et al., 1999; Hosoda et al., 2000b; Hosoda et al., 2000a; Hosoda et al., 2003). Kirchner et al. (2009) proposed that the availability of acyl groups and relative concentration in gastric tissues may account for the skewed n-octanoylated ghrelin secretion. In support of this theory, data presented by these researchers indicated a correlation between the type of dietary lipid substrate (C8:0 or C10:0) available from ingested feed and a change in the ratio of C8:0 to C10:0 modified ghrelin peptides (Kirchner et al., 2009).

Nonetheless, though the fatty acid substrates for gastric ghrelin acylation were shown to derive directly from ingested feed, the origin of medium-chain-fatty acids supplied to non-gastrointestinal GOAT-expressing tissues has yet to be determined (Lim et al., 2011b).

Hosoda et al. (2003), have also isolated several forms of ghrelin from human plasma and have classified the peptides into four groups based on the type of acyl modification on Ser3. The four classes of ghrelin are: nonacylated, octanoylated (C8:0), decanoylated (C10:0), and decenoylated (C10:1). In addition, ghrelin peptides may either have 27 or 28 amino acids. The 27-amino-acid-long ghrelin derives from a C-terminal truncation of Arg28 that is most likely removed via carboxypeptidase hydrolysis. Although the 28-amino acid Ser3-octanoylated ghrelin is the most highly expressed in humans, the minor forms of acylated ghrelin are also biologically functional and can stimulate GHSR signal transduction in cell cultures as well as GH release in rats (Hosoda et al., 2003; Kojima and Kangawa, 2010).

GOAT Substrate Specificity- Ghrelin Binding pocket

Amino acid substitution mutations for both ghrelin and GOAT have been conducted to better understand the critical residues required for ghrelin acylation. Ghrelin inter and intra-species variants differ in amino acid length and composition however, all share a highly conserved N-terminal sequence. Recognition of ghrelin as a substrate for GOAT may be based on the first seven amino acids for which few point mutations have occurred during evolution. The first seven amino acids of all ghrelins identified (NCBI database, http://www.ncbi.nlm.nih.gov/) are GlySerSerPheLeuSerPro with a few exceptions: Gly**Thr**SerPheLeuSerPro (zebrafish, CAJ20254), Gly**LeuThr**PheLeuSerPro (bullfrog, BAB71718), and Gly**Thr**SerPheLeuSerPro (xenopus frog, BAL70270). Interestingly, however, although the ghrelin N-terminal sequence is highly conserved, mutations of ghrelin Ser2, Leu5,

Ser6, or Pro7 with Ala do not impede GOAT activity (Yang et al., 2008a; Yang et al., 2008b). Only when Gly1, Ser3, or Phe4 are substituted with Ala, the result is a loss of proper ghrelin acylation (Yang et al., 2008b). In bullfrogs an evolutionary point mutation of Ser3 to Thr has led to a fully functional isoform of ghrelin (Kaiya et al., 2001) and when Ser3 is replaced with Thr in rat ghrelin, rat GOAT transferase activity remains optimal, hence, indicating that either serine or threonine is acceptable at the third amino acid position (Yang et al., 2008a; Yang et al., 2008b).

Mutations of GOAT have also been conducted and substitutions of highly conserved residues Asp307 or His338 (numbering based on mouse GOAT) with Ala render GOAT incapable of acylating ghrelin (Gutierrez et al., 2008; Yang et al., 2008a). Thus, for ghrelin the critical amino acid residues are Gly1, Ser3 or Thr3, and Phe4 while for GOAT the critical residues are Asp307 and His338. At this time, a crystal structure of GOAT has not been published to further clarify critical amino acid interactions responsible for substrate specificity at the CoA-fatty acid and ghrelin binding pockets (www.rcsb.org/pdb).

Regulation and Physiological Effects of the Ghrelin-GOAT System

In trials conducted on human gastric and enteric tissues, Lim et al. (2011a) noted that no direct quantitative correlation could be observed through qrtRT-PCR analyses of ghrelin and GOAT mRNA expression. These differences may be indicative of independent regulatory mechanisms for synthesis. However, during fasting, gastric ghrelin mRNA as well as total plasma ghrelin concentrations increase in both human and murine models (Cummings et al., 2001; Toshinai et al., 2001) and Gahete et al. (2010) reported that stomach GOAT expression also increased when mice were fasted. On the other hand, in a study conducted by Kirchner et al. (2009), gastric GOAT decreased during fasting but was highest in ad libitum fed mice provisioned with diets rich in medium-chain fatty acid. Thus, the researchers proposed that the

ghrelin-GOAT system was downregulated when fatty acid substrates could not be derived from the diet for ghrelin acylation (Kirchner et al., 2009).

Natural diurnal fluctuations in plasma ghrelin levels have also been reported to increase pre-prandially and decrease post-prandially further supporting the idea that ghrelin-GOAT axis is correlated to food availability (Cummings, 2006). The importance of nutritive food rather than mechanical distention of the stomach has been emphasized by the lack of response observed in plasma ghrelin levels of mice and rats subjected to gastric distention with non-nutritive water (Tschop et al., 2000; Williams et al., 2003). On the other hand, the extent to which ghrelin secretion is affected by a meal is dependent on the nutrient composition. For example, ingesting a meal high in lipids suppress total plasma ghrelin levels poorly in comparison to a carbohydrate dense meal. This phenomenon has been observed in humans as well as rodent models (Sanchez et al., 2004; Cummings, 2006). Similarly, in comparison to pair-fed rats provisioned with high fat or high carbohydrate diets, rats fed protein-rich diets not only exhibit higher ghrelin plasma levels once fasting is initiated but also after re-feeding begins (Vallejo-Cremades et al., 2004).

The Avian Ghrelin-GOAT System

Avian Ghrelin

Avian ghrelin was cloned in 2002 by Kaiya et al. Avian preproghrelin is composed of 116 amino acids and shares very little amino acid sequence homology with mammalian preproghrelin except in the core UAG sequence (Yuan et al. 2007). Similar to the processing of human ghrelin, the first 23 amino acids of chicken preproghrelin are cleaved to yield proghrelin. Although the chicken proghrelin sequence has no amino acid deletions in the 28 amino acid DAG core sequence that follows the signal sequence, it is processed differently than human

ghrelin. N-terminal sequencing of isolated chicken ghrelin reveals that it consists of only 26 amino acids (Kaiya et al. 2002). When DAG is cleaved from proghrelin the amino acid residues in position 27 and 28 are left with the proghrelin portion of the protein (Kaiya et al. 2002, Yuan et al. 2007). As in mammalian species, it is the third amino acid residue, a serine, which is acylated (Kaiya et al. 2002).

The tissue distribution pattern of avian ghrelin mRNA and protein expression is similar to that of mammalian species. The highest expression levels are found in the proventriculus, the glandular portion of the avian stomach, followed by the small intestines (Kaiya et al. 2002, Wada et al. 2003, Richards et al. 2005). Avian ghrelin mRNA is also expressed in the pancreas, adipose tissue, lung, spleen, and brain, but at levels lower than what is detected in the digestive tract (Kaiya et al. 2002, Wada et al. 2003, Richards et al. 2005, Kaiya et al. 2007). It is assumed that the proventriculus is the main source of circulating ghrelin in avian species (Richards et al. 2005, Kaiya et al. 2007).

Avian Ghrelin Receptor

Avian GHSR was characterized in 2003 by two separate research groups (Geelissen et al., 2003; Tanaka et al., 2003). Both groups reported that chickens have a GHSR gene structure analogous to that seen in humans with the avian GHSR gene being composed of two exons. The GHSR1a mRNA sequence in chickens codes for a protein of 347 amino acids. Alternate splicing of the GHSR transcript yields two other forms of the chicken ghrelin receptor, GHSR1aV and GHSRtv (Tanaka et al., 2003; Sirotkin et al., 2006). GHSR1aV lacks the first 16 amino acids coded by exon 2 and thus is predicted to lack transmembrane region 6 (Tanaka et al., 2003) and to have its C-terminal region located on the extracellular side of the cell membrane. The GHSR1tv transcript forms from a premature splicing from exon 1, retention of a 126 bp fragment

from intron 1, and premature initiation of exon 2, all of which results in a shift in the open reading frame of the message that results in a new stop codon at amino acid 221. It is unclear if both of these truncated forms of the chicken GHSR receptor are even translated from their altered mRNA sequences (Sirotkin et al., 2006). GHSR1a mRNA expression in avian species is very high in the hypothalamus and pituitary, and lower levels of expression are found in the proventriculus, duodenum, adrenals, ovary, testes, liver, muscles, heart, and skin (Geelissen et al., 2003; Richards et al., 2006). The mRNA for the ghrelin receptor has been detected in the theca and granulosa cells from hierarchical and nonhierarchical follicles and the mRNA expression of the ghrelin receptor is down-regulated by FSH and LH in cultured granulosa cells (Freeman, 2008).

In 2006, Richards et al reported that broilers fasted for 48 hours had increased ghrelin mRNA levels in the proventriculus, but that plasma concentrations of total ghrelin remained unchanged. In addition, ghrelin mRNA expression remained high in the proventriculus 12 hours after the birds had been re-fed. Rodents, on the other hand, have an increase in both ghrelin mRNA levels in the stomach and plasma levels of total ghrelin when they are fasted and both decrease within 6 hours after re-feeding (Toshinai et al., 2001). Research reports in quail and male Leghorn chicks, in which acylated ghrelin was specifically measured, indicate that plasma ghrelin levels increased and subsequently decreased upon re-feeding (Shousha et al., 2005; Kaiya et al., 2007). However, it was again noted by Kaiya et al. (2007) that the level of ghrelin mRNA in the proventriculus remained high 24 hours after the Leghorn chicks had been re-fed. Plasma acylated ghrelin levels also increase in broiler breeder hens that are fasted (Freeman, 2008).

Similar to mammalian species, ghrelin has been found to be a potent *in vivo* and *in vitro* stimulator of growth hormone release in Leghorn chickens (Ahmed and Harvey, 2002; Baudet

and Harvey, 2003). However, ghrelin's effects upon feeding behavior are not as clearly defined in avian species as they are in rodents and humans. Since the discovery of ghrelin in avian species, there has been only one report of peripherally injected ghrelin stimulating feeding behavior in birds and that occurred in adult quail (Shousha et al., 2005). Intracerebroventricular injection of ghrelin into broiler chicks or peripheral injection of ghrelin into Leghorn chicks inhibited (Furuse et al., 2001; Saito et al., 2002; Saito et al., 2005) or had no effect on feed intake (Kaiya et al., 2007). Kaiya et al. (2007) reported that peripheral ghrelin administration caused a decrease in plasma glucose levels in 8-day old male laying chicks. On the other hand, peripherally injected ghrelin did not have any effect on plasma glucose, triglyceride, free fatty acids, protein, or T3 levels in week-old male Ross broiler chicks (Geelissen et al., 2006).

Avian Ghrelin O-acyltransferase

There has been no characterization of avian GOAT other than an identification of a putative gene for avian GOAT based on the publication of the chicken genome (Yang et al., 2008a).

Summary

In mammals, ghrelin is predominantly produced by the glandular gastric tissue, and is found in two forms: DAG and acylated ghrelin. The latter form is covalently modified with a fatty acid moiety, which is catalyzed by the ER associated, membrane bound enzyme, GOAT. The acyl moiety is essential for affinity between the ghrelin peptide and it's endogenous receptor, GHSR. Like its substrate, ghrelin, GOAT is also highly conserved across vertebrates. In mammals, the ghrelin-GOAT system has been studied extensively and has been implicated in mediating the signal for nutrient intake between the peripheral tissues of the body and the CNS. In avian species, the ghrelin system is also implicated in regulation of energy homeostasis

although more research is needed to understand the functions of ghrelin in birds. Similarly to mammals, avian ghrelin is prominently produced by the glandular stomach, the proventriculus, however, in contrast to mammalian ghrelin, ghrelin in birds may act as an anorectic hormone rather than inducing feed intake. In addition, avian GOAT has not been studied and further research is warranted to elucidate the functions of the ghrelin-GOAT axis in avian species.

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CHAPTER 3

Statement of Purpose

It is becoming more common for broilers to be raised to 7 weeks of age and older in order to gain the breast yield desired for the further processed specialty markets. The live weight of these broilers will often exceed 3.5 kilograms. These broilers are typically provided a withdrawal diet from 5 weeks of age until market age. Surprisingly little research (1 published manuscript) has been conducted on the amino acid requirements for optimal growth performance and efficiency for broilers during this period. The Nutritional Research Council requirements for poultry were last published in 1994 when broilers achieved a body weight of less than 3 kg at 7 weeks of age. These guidelines provided nutrient requirements for broilers from 0 to 3, 3 to 6, and 6 to 8 weeks of age. Experimental data was lacking for many of the amino acid requirements in the 6 to 8 week category. Thus, there is no set guideline for dietary amino acid concentrations in the current critical age period of 5 to 7 weeks. Ongoing amino acid requirement research is essential because genetic strain improvements in broilers continue with regard to feed efficiency and meat yield. In addition, amino acid requirement patterns change as more synthetic amino acids become available allowing for an overall reduction in the level of crude protein fed. Furthermore, amino acid requirements can vary based on environmental conditions such as heat stress. In broiler diets, the lysine requirement is critical as lysine is typically the second most limiting amino acid in typical corn-soy based diets and is utilized more heavily than the other amino acids in muscle synthesis. Threonine is typically the third most

limiting amino acid in poultry diets and it is starting to become widely available for use in commercial poultry diets. Although great strides have been made in optimizing broiler performance, continued improvement will result from a better understanding of the regulatory mechanisms involved in feed intake and energy metabolism. Ghrelin, a protein hormone that was first discovered in 1999 in mammalian species and 2002 in chickens, has been shown to regulate feeding behavior and energy metabolism. However, the addition of an acyl moiety is essential for ghrelin to bind to its cognate receptor, and this is accomplished by the enzyme GOAT. While ghrelin and the ghrelin receptor have been identified and studied in the chicken, GOAT has not been studied. Therefore, the goals of the present research are to 1) characterize the digestible lysine requirement in two commonly grown genetic crosses of broilers from 35 to 49 days of age, 2) to determine the digestible lysine requirement of heat stressed broilers from 14 to 28 days of age, 3) to determine the digestible threonine requirement of broilers from 14 to 28 days of age and 4) to characterize the mRNA expression of GOAT in broilers.

CHAPTER 4

DIGESTIBLE LYSINE REQUIREMENT OF COBB X COBB 500 FF MIXED-SEX BROILERS AND COBB X HUBBARD M99 MALE BROILERS FROM THIRTY-FIVE TO FORTY-NINE DAYS OF AGE[1]

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ABSTRACT

Defining the digestible lysine (**dLys**) requirement of modern broiler strains is important for optimal breast meat production. Two experiments were conducted to determine the dLys requirements of mixed-sex Cobb x Cobb 500 fast-feathering (**FF)** broilers as well as male Cobb 500 FF female x Hubbard M99 male crossbred broilers during the withdrawal period (35 to 49 days of age). In each experiment, day-of-hatch chicks were randomly allotted to 96 floor pens (4 rooms, 24 pens per room) and fed the same starter (0 to7 days), grower (7 to 21 days), and finisher (21 to 35 days) diets which were formulated to meet or exceed NRC requirements. During the withdrawal period, 12 replicate pens of birds were fed one of 8 dietary treatments. The treatment diets were derived from a common base diet and common summit diet. The common base diet supplied 3,215 kcal/kg, 13.28% crude protein (**CP**), and 0.60% dLys for experiment 1 and 3,215 kcal/kg, 13.71% (CP), and 0.62% dLys for experiment 2. The common summit diet supplied 3,215 kcal/kg, 22.00% CP, and 1.16% dLys for experiment 1 and 3,215 kcal/kg, 23.59% CP, and 1.24% dLys for experiment 2. By blending the base and summit diets, 6 intermediate levels of dLys and dietary CP were produced for experiments 1 and 2, respectively. Minimum ideal essential amino acid ratios were maintained across the 8 diets. Broken-line regression analyses estimated the withdrawal phase dLys requirement as a % of diet for body weight gain, feed conversion, and total white meat to be 0.96, 0.99, and 0.95 for Cobb x Cobb mixed-sex broilers and 0.86, 0.91 and 0.90 for Cobb x Hubbard male broilers, respectively. These studies aid in defining the dLys requirement in 35-49 day-old broilers.

KEYWORDS: broiler performance, meat yield, amino acid requirement

INTRODUCTION

The broiler industry uses genetic selection and advancements in nutrition and management practices to improve body weight gain (**BWG**), feed conversion (**FC**), cost of production, and the accretion of breast muscle. Of the nutrients that are required by the rapidly growing broiler, the dietary level of amino acids and their relative ratio to digestible lysine (**dLys**) largely determine the bird's ability to efficiently synthesize body protein. Essential amino acid (**AA**) requirements for broilers have been well documented for many years. However, continuous genetic selection for larger and faster growing birds has perpetually increased dietary protein inclusion needs for optimal broiler performance (Sibbald and Wolynetz, 1986; NRC, 1994; Havenstein et al., 2003b; Havenstein et al., 2003a; Kidd et al., 2004; Dozier et al., 2008b). Currently, synthetically manufactured DL-methionine, L-lysine, L-threonine, L-tryptophan, and L-valine are commercially available as feed additives. Accessibility to supplemental commercial AA allows for reduction of dietary crude protein (**CP**) incorporation while improving diet formulation flexibility to better fit the changing AA requirement patterns of birds depending on factors such as flock age, sex, genetic strain, and grow-out environmental conditions (Han and Baker, 1993; Corzo et al., 2003; Corzo et al., 2006; Garcia et al., 2006; Dozier et al., 2010).

In the Untied States, high consumer demand for white meat has led the poultry industry to genetically select birds with larger breast muscles. As reviewed by Dozier et al. (2008b), the price of breast meat impacts gross feeding margins more profoundly than the price of feed ingredients. Thus, synthetic AA inclusion levels should be set accordingly after assessing both market factors. Of the ten essential AAs, Lys has the greatest impact on breast muscle development due to its high percentage of incorporation into muscle structures relative to other

amino acids (Munks et al., 1945). Tesseraud et al. (1996) demonstrated that dietary Lys is essential for breast muscle formation and that Lys deficiency in chicks leads to significant total white meat yield reduction. Furthermore, with the genetic selection for increased breast muscle yield in broilers grown to 49 days of age, the Lys requirement has likely increased in these birds. In a 49 day grow-out period, the growing broiler will consume just over 50% (Cobb-Vantress, 2008a) of its total feed intake during days 35 through 49 when reared from 0 to 49 days of age. Thus having the correct dietary dLys requirement is critical during this period. However, most of the research on the Lys requirement of broilers has been focused on the requirement before or up to day 35 of age.

In the United States broiler market, the Cobb 500 strain is widely used; but in recent years, the number of broiler progeny derived from a Cobb x Hubbard cross has increased (Agristats, 2010). Due to the relatively recent emergence of this cross, the dLys requirement for these birds has yet to be established. The objective of the current research was to determine the dLys requirement for optimal performance during the withdrawal period form 35 to 49 days of age of broiler progeny from two genetic crosses: 1) Cobb x Cobb 500 fast feathering (**FF**) and 2) Cobb 500 FF female x Hubbard M99 male.

MATERIALS AND METHODS

Bird Husbandry

Two experiments were conducted in a facility with 4 identical but separate rooms where each room had 24 (3.05 m by 1.22 m) floor pens. All pens were equipped with 7 nipple drinkers originating from a common water line and 1 pan feeder (0.09 m^2). All stocking density calculations accounted for the area occupied by the pan feeders. Prior to chick placement, litter that had been used by 2 previous flocks was top-dressed with fresh pine shavings. A continuous lighting program was implemented with a light intensity of 20 lux for 24 hours (0 to 4 days), 20 lux for 20 hours (5 to 7days), 10 lux for 16 hours (8 to 14 days), and 2 lux for 16 hours (15 to 42 days). The light intensity was maintained at 2 lux from day 43 to 49; however, the hours of light were extended to 23 hours per day. Light intensity was verified by placing a Light ProbeMeter™ (model 403125, Extech Instruments Corp. Waltham, MA) into the pens. Management protocols were followed according to the Cobb brooding and broiler management guidelines (Cobb-Vantress, 2008a and 2008b).

For each room, a computerized controller regulated 2 gas-fired furnaces, an exterior evaporative cooling system present on both sides of the room for intake air four 45.7 cm ceiling circulation fans, and for air clearance at the end of each room two 91.4 cm exhaust fans and one 61cm exhaust fan. Ambient temperature was set to 34 °C on day 1 and decreased by 0.28 °C until 24 °C was reached and then maintained. No significant differences in temperature and humidity were noted throughout the studies between the 4 rooms. Vaccinations for Marek's disease, Newcastle disease, and infectious bronchitis were administered at the hatchery. All animal procedures were approved by the University of Georgia Animal Care and Use Committee, Athens, GA.

Experiment 1

Prior to placing chicks, the 96 pens were assigned to one of 8 dietary withdrawal treatments in a random block design (12 replicates per treatment, 3 replicate pens per treatment per room). A total of 5,400, day of hatch, Cobb 500 x Cobb FF broiler chicks originating from the same breeder flock were purchased from a primary breeder hatchery. The chicks were sorted and those with extreme weights were discarded before the remaining birds were assigned to the 96 pens (50 birds per pen). On day 35, the start of the dietary treatment phase, bird density was equalized to 46 birds per pen (23 males and 23 females based on visually sexing the birds; 0.081 m^2/bird) so that differences in performance due to bird quality or gender were minimized.

Experiment 2

Experiment 2 followed the same protocol as experiment 1 except that only male day of hatch progeny from a Cobb 500 FF female x Hubbard M99 male cross were selected and used. On day 35, the birds were weighed and reduced to 42 birds per pen (0.089 m^2/bird) to account for the larger body mass associated with using all male birds in this experiment.

Dietary Treatments

Feed and water were provided ad libitum for each period: starter (0 to 7 days of age, crumble diet), grower (7 to 21 days of age, pelleted diet), finisher (21 to 35 days of age, pelleted diet), and withdrawal (35 to 49 days of age, pelleted diet). The common starter, grower, and finisher diets (Table 4.1) were formulated to meet or exceed NRC (1994) requirements. The base and summit withdrawal diets (Table 4.2) were used to create 6 intermediate withdrawal diets (Table 4.3). Minimum digestible EAA:dLys ratios were maintained in the base and summit diets to ensure that the diets contained an adequate level of essential amino acids and that the response to dLys would not be limited due to amino acid interactions. The corn, soybean meal

and porcine meat and bone meal used in the diets were analyzed for nutritional content prior to formulation (total amino acid content, crude protein, and minerals) and digestible amino acid values were determined using the cecectomized rooster assay.

Measurements

For each room, humidity, temperature, water consumption, and pen mortality were recorded daily. Birds and feed were weighed on days 0, 7, 14, 35, and 49 to determine BW, feed intake (FI), BWG, and FC. On day 49, the mean bird weight for each pen was determined and 8 birds (4 males and 4 females in experiment 1, and 8 males in experiment 2) per pen within 300 g above/below the mean weight of their pen were selected for processing. Individual weights for the selected birds were recorded and each bird was leg banded prior to placement in a coop for an overnight feed withdrawal before processing. On day 50, birds were weighed and processed at the University of Georgia's Pilot Processing Plant as previously described (Hidalgo et al., 2004). During evisceration the abdominal fat pad surrounding the intestinal cavity and extending through the proventriculus was stripped from the visceral organs and weighed for each bird. Subsequently, eviscerated hot carcass weights were recorded for each bird prior to static chilling for 24 hours. On day 51, two professional deboners from a local processing plant performed cut up. Chilled carcasses were drained and each deboner was allocated one-half of the carcasses from each of the 8 treatments. Weights were recorded for: drained chilled carcass, *pectoralis major*, *pectoralis minor*, wings, and leg quarters of each bird. Percent yield calculations were based on the fasted, live weight of the bird.

Statistical Analyses

ANOVA using the GLM was used to evaluate the completely randomized block design and the room effect was not significant. In addition, on day 35, ANOVA using GLM was used

to determine that no significant differences in BW existed across the proposed experimental dietary treatments. ANOVA was completed using Minitab (Release 13, State College, PA). Live production and processing data were subjected to broken line regression analysis to determine dLys requirements (Vedenov and Pesti, 2007).

RESULTS

Experiment 1

For the Cobb x Cobb 500 FF mixed-sex broilers, the initial average BW per pen on day 35 was 2,148 g/bird. Variability in the mean starting BW between treatments was not significantly different (CV = 2.69 %, P = 0.9624). A significant and positive response to the increasing dLys levels was observed for BW, BWG, and FI, (Table 4.4). A similar trend was seen with the processing parameters measured with yield increasing and abdominal fat decreasing with increasing levels of dLys and protein (Table 4.5). Using the linear broken-line model, the dLys requirement as percent of diet ranged from 0.79 for chilled carcass weight to 1.00 for fat pad weight (Table 4.6). The total dLys requirement per bird from 35 to 49 days of age ranged from 20.93 g for fat pad to 22.30 for total white meat yield (Table 4.6). Because total crude protein levels changed in the experimental diets in order to maintain EAA ratios, the crude protein requirement was also calculated and ranged as a percent of diet from 16.26 for BWG to 21.42 for total white meat yield (Table 4.6).

Experiment 2

On day 35, the initial pen average BW for male broilers from the Cobb 500 FF female x Hubbard M99 male cross was 2,355 g/bird with a CV of 0.48%. Differences in the initial start weight of the birds across the treatments were not significantly different (P = 0.7804).

Paralleling experiment 1, increasing dLys levels caused significant and positive responses in BW, BWG and FI while increasing dLys levels lowered FC values (Table 4.7). Total breast meat yield increased and abdominal fat content decreased as the level of dLys increased (Table 4.8). Using the linear broken-line model, the dLys requirement as percent of diet ranged from 0.86 for BW and BWG to 1.07 for FI (Table 4.9). The total dLys requirement per bird from 35 to 49 days of age ranged from 24.45 g for BWG to 30.66 grams for leg quarter yield (Table 4.9). Total crude protein requirements as a percent of diet ranged from 17.61 for BWG to 23.53 for BW (Table 4.9).

DISCUSSION

Due to genetic selection, today's broiler has higher essential amino acid requirements compared to those in the past as it consumes less feed per unit of BWG and has greater muscle accretion (Dozier et al., 2008a; Dozier et al., 2009; Dozier et al., 2010). In the current research, the percent of dietary dLys requirement based on averaging the determined requirement for BWG, FCR, and total white meat yield was 0.966% for mixed sex Cobb x Cobb 500 FF broilers and 0.890% for male Cobb 500 x Hubbard M99 broilers during the finishing period from 35 to 49 days of age. The results from both studies cannot be directly compared as the Cobb x Hubbard flock consisted of all male progeny while the Cobb x Cobb 500 FF flock was a mixed-sex flock. Thus, the Cobb x Cobb 500 FF broiler dLys requirement analyses were skewed by the presence of female broilers, which consume less feed, have lower AA requirements, poorer FCR and lower meat yield when compared to their male counterparts (Han and Baker, 1994; Mack et al., 1999; Baker et al., 2002; Rostagno et al., 2007; Dozier et al., 2008a; Dozier et al., 2009).

The only other published report on the dLys requirement during this equivalent age period was conducted using Ross male broilers and the requirement was determined to be 1.10% or greater (Neto et al., 2009). The difference between this previous result and the current ones is not clear. In the present research the initial starter, grower and finisher diets were designed to be overly adequate in all amino acids so that the birds would not be limited in size or response to the dietary treatments in the experimental withdrawal phase.

Research completed on the dLys requirement during the period from 28 to 42 days of age in male Ross x Ross TP16 broilers and male Cobb x Cobb 700 broilers determined a requirement based on BWG, FCR and total white meat yield of 0.988% and 1.001%, respectively (Dozier et al., 2010). The requirement in male Ross x Ross 708 broilers from 49 to 63 days of age based on the same three parameters was 0.88% (Dozier et al., 2008a). The dLys requirements determined for the two genetic crosses in the current research from 35 to 49 days of age, as expected, fell between the requirements calculated for the younger and older birds in the previous research. In addition, based on percent of diet, the broilers from the Cobb 500 FF hens x Hubbard M99 rooster cross, yields a very efficient broiler with potentially a slightly lower dLys requirement for optimal performance relative to other popular broiler crosses.

In the current research, the broilers from both genetic strains exhibited higher dLys requirements as a percent of diet for FCR compared to BWG. For the Cobb 500 mixed-sex birds, the dLys requirement for FCR was estimated to be 0.99% and 0.96% for BWG while the dLys requirement for the Cobb x Hubbard male broilers was estimated to be 0.91% and 0.86% for FCR and BWG, respectively. Previously, the dLys requirement for FCR has also been found to be notably higher than the requirement for BWG (Han and Baker, 1993; Leclercq, 1998; Mack

et al., 1999; Baker et al., 2002; Dozier et al., 2009). But, it should be noted that when calculated on a gram basis to account for feed intake, the dLys requirement for FCR exceeds that of BWG.

Two differing experimental approaches can be utilized in determining the requirement of a specific amino acid. In the most commonly used approach for broilers, graded levels of a commercially synthesized single amino acid are added to a basal diet deficient in the amino acid in question and the response is determined. In this method, the nutritionally complete diet has a fixed crude protein level except for the amino acid being tested. Thus, as the level of the amino acid being tested increases, the ratio of this amino acid to the other amino acids changes. The balance of amino acids, in particular essential amino acids, is critical as it is well known that amino acid interactions such as imbalances and antagonisms effect the requirement of a given amino acid whether the diets are protein deficient or protein adequate (Harper et al., 1970; Hapner, 1976; Davis and Austic, 1982a; Davis and Austic, 1982b; Esteve-Garcia, 1984; Robbins, 1987). In addition, once the graded increment of the tested amino acid reaches its requirement, further BWG may be inhibited by the existence of another amino acid in the diet becoming the most limiting. These effects have been documented for lysine in poultry numerous times (Grau, 1948; Morris et al., 1987; Abebe and Morris, 1990; Surisdiarto and Farrell, 1991; Plumstead et al., 2007). Therefore, another approach, and the one used in the current research, is to maintain the balance (ratio) of essential amino acids as the level of the essential amino acid in interest is increased in graded increments. To accomplish this, the dietary level of protein also increases in concert. The downfall of this method is that the requirement of the amino acid for a parameter could be based on a protein effect or the amino acid being tested, as will be discussed below for abdominal fat deposition. However, given that lysine is the basis for setting the ratios

of all other essential amino acids in the ideal amino acid formulation concept, we chose to keep the essential amino acid ratios balanced in the current research.

The decrease in the percent body fat yield with increasing dietary lysine and protein level was as expected in both experiments. A decrease in the amount of abdominal fat in broilers as dietary protein levels increase is well documented (Cabel et al., 1988; Cabel and Waldroup, 1991; Summers et al., 1992; Deschepper and De Groote, 1995; Smith and Pesti, 1998; Sklan and Plavnik, 2002; Chendrimada et al., 2007). Research indicates that this is due to a decrease in de novo fatty acid synthesis and, in particular, a decrease in the activity of malic enzyme activity which produces the NADPH necessary for de novo fatty acid synthesis (Tanaka et al., 1983; Grisoni et al., 1991; Adams and Davis, 2001). The decrease in malic enzyme activity results from lower expression of the enzyme due to a decrease in mRNA transcription of the gene and interestingly this decrease in transcription can be caused by an increase in dietary protein, specific amino acids or even non-protein nitrogen (Adams and Davis, 2001; Chendrimada et al., 2007).

In summary, the dLys requirement as a percent of diet was 0.966% for mixed sex Cobb x Cobb 500 FF broilers and 0.890% for male Cobb 500 x Hubbard M99 crossbred broilers during the finishing period from 35 to 49 days of age. The results indicate that the 35 to 49 day old broiler is responsive to higher levels of dietary dLys than current NRC recommendations. In addition, with balanced amino acid ratios the crude protein requirement for BWG was lower than that for total white meat in both experiments, but as always, the decision to obtain incremental gains in meat yield beyond the requirements for BWG has to balanced with cost effectiveness.

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Table 4.1. Composition of diets for the starter, grower, and finisher phases, experiments 1 and 2

	Experiment 1			Experiment 2		
Ingredient	Starter[4]	Grower[5]	Finisher[6]	Starter[4]	Grower[5]	Finisher[6]
	----------(%) ----------					
Corn	53.777	57.443	58.600	53.650	56.182	57.552
Soybean meal, 48.0% CP	37.986	32.595	30.555	33.886	30.807	29.767
Meat and bone meal	3.000	5.000	5.019	3.598	3.017	2.383
Peanut meal	-	-	-	3.500	3.500	3.500
Poultry Oil	2.472	3.115	4.297	1.800	3.263	3.888
DDGS	-	-	-	1.000	0.750	0.500
Limestone	0.601	0.413	0.370	0.989	0.996	1.003
Dicalcium Phosphate	1.090	0.305	0.100	-	-	-
Salt	0.488	0.590	0.560	0.754	0.734	0.715
DL-Met	0.318	0.285	0.268	0.352	0.311	0.288
L-Lys	0.051	0.057	0.061	0.187	0.171	0.153
L-Thr	-	-	-	0.063	0.063	0.052
Phytase[1]	-	-	-	0.020	0.020	0.020
Trace mineral mix[2]	0.062	0.063	0.050	0.063	0.063	0.063
Vitamin mix[3]	0.025	0.025	0.020	0.025	0.025	0.025
Monteban-45	0.070	-	0.060	0.063	-	0.054
Choline chloride 60%	0.020	0.020	0.020	0.020	0.020	0.020
BMD-50	0.040	0.040	0.020	0.036	0.036	0.018
Coban-90	-	0.050	-	-	0.045	-

[1]Quantum 2500 XT (AB Vista, NC)

[2]Trace mineral premix provides the following in milligrams per kilogram of diet: manganese, 176; zinc, 176; iron, 64; copper, 8.8; iodine, 2.8; selenium, 0.5.

[3]Vitamin premix provides the following per kilogram of diet: vitamin A, 7,000 IU; vitamin D_3, 2,500 IU; vitamin E, 19 IU; vitamin K, 1.3 mg; vitamin B_1, 1.6 mg; vitamin B_2, 6.3 mg; vitamin B_6, 2.4 mg; vitamin B_{12}, 0.01 mg; niacin, 40 mg; pantothenic acid, 11 mg; folic acid, 0.7 mg; biotin, 0.08 mg.

[4]Starter diet fed from 0 to 7 days of age (crumble diet).

[5]Grower diet fed from 7 to 21 days of age (pelleted diet).

[6]Finisher diet fed from 21 to 35 days of age (pelleted diet).

Table 4.2. Composition of withdrawal treatment base and summit diets provided to broilers from 35 to 49 d-of-age, experiments 1 and 2

	Experiment 1		Experiment 2	
Ingredient	Base Diet	Summit Diet	Base Diet	Summit Diet
	---------- (%) ----------			
Corn	80.95	58.18	82.08	51.210
Soybean meal, 48.0 % CP	10.12	32.64	13.10	38.420
Meat and bone meal	2.780	2.780	2.590	2.590
Wheat Middlings	3.450	-	-	-
Poultry Oil	1.000	4.590	0.550	5.900
Salt	0.574	0.612	0.160	0.670
Limestone	0.481	0.354	0.750	0.590
DL-Met	0.074	0.334	0.080	0.350
L-Lys·HCl	0.108	0.138	0.090	0.100
L-Thr	0.000	0.086	-	0.070
Dicalcium Phosphate	0.101	0.214	-	-
Phytase[1]	-	-	0.020	0.010
Trace mineral mix[2]	0.050	0.050	0.060	0.060
Vitamin mix[3]	0.020	0.020	0.030	0.030
Sodium -Carbonate	-	-	0.400	-
Choline Chloride 60%	0.088	0.020	0.110	0.020
ME, kcal/kg	3215	3215	3215	3215
CP, %	13.28	22.00	13.71	23.59
Analyzed CP, %	14.35	23.71	13.53	23.46
Total Lys, %	0.68	1.24	0.70	1.38
Thr	0.49	0.87	0.71	0.36
Met	0.35	0.64	0.94	0.53
Avail. P, %	0.38	0.38	0.38	0.38
dThr:Lys	66.00	66.00	68.00	68.00
dMet:Lys	46.46	54.20	47.84	54.87
dTSAA:Lys	76.00	76.00	77.00	77.00
dArg:Lys	112.28	109.45	122.53	118.16
dIle:Lys	82.80	74.45	74.86	71.55
dLeu:Lys	198.27	145.92	185.68	139.97
dVal:Lys	89.32	76.00	80.69	78.00
dTrp:Lys	16.48	18.00	18.49	19.31
tGly:dLys	109.80	88.39	111.82	89.05

[1]Quantum 2500 XT (AB Vista, NC)

[2]Trace mineral premix provides the following in milligrams per kilogram of diet: manganese, 176; zinc, 176; iron, 64; copper, 8.8; iodine, 2.8; selenium, 0.5.

[3]Vitamin premix provides the following per kilogram of diet: vitamin A, 7,000 IU; vitamin D_3, 2,500 IU; vitamin E, 19 IU; vitamin K, 1.3 mg; vitamin B_1, 1.6 mg; vitamin B_2, 6.3 mg; vitamin B_6, 2.4 mg; vitamin B_{12}, 0.01 mg; niacin, 40**.**

Table 4.3. Blending of withdrawal diets, experiments 1 and 2

Treatment	Experiment 1				Experiment 2			
	dLys	CP	Base Diet	Summit Diet	dLys	CP	Base Diet	Summit Diet
	---------- (%) ----------				---------- (%) ----------			
1 (Base Diet)	0.60	13.28	100.00	-	0.62	13.71	100.00	-
2	0.68	14.52	85.71	14.29	0.71	15.12	85.71	14.29
3	0.76	15.77	71.43	28.57	0.80	16.53	71.43	28.57
4	0.84	17.02	57.14	42.86	0.89	17.94	57.14	42.86
5	0.92	18.26	42.86	57.14	0.97	19.35	42.86	57.14
6	1.00	19.51	28.57	71.43	1.06	20.77	28.57	71.43
7	1.08	20.76	14.29	85.71	1.15	22.18	14.29	85.71
8 (Summit Diet)	1.16	22.00	-	100.00	1.24	23.59	-	100.00

Table 4.4. Live performance of Cobb 500 x Cobb 500 FF mixed sex broilers fed progressive concentrations of digestible lysine (dLys) from 35 to 49 days of age, experiment 1[1]

dLys	FI	BW	BWG	FCR[2]	Mortality
(%)	---------- g / bird ----------			g / g	(%)
0.60	2462	2927	713	3.12	4.50
0.68	2338	2951	771	2.96	1.45
0.76	2368	3025	827	2.75	1.99
0.84	2250	2999	766	2.71	3.45
0.92	2267	3049	868	2.57	2.36
1.00	2204	3050	856	2.43	2.17
1.08	2201	3069	915	2.37	1.27
1.16	2193	3064	849	2.42	2.90
SEM	43	43	34	0.05	0.71
Source of variation	---------- P- value ----------				
Linear	0.000	0.000	0.000	0.000	NA[3]
Quadratic	0.000	0.005	0.008	0.000	NA

[1]Values are means of 12 replicate pens per treatment, each with 46 birds (23 males, 23 females) per pen from 35 to 49 days of age.
[2]Values represent feed consumed per bird divided by body weight gain (BWG) corrected for mortality.
[3]Mortality data were not subjected to breakpoint regression analysis, thus, P-values were not applicable (NA).

Table 4.5. Processing yield responses of Cobb 500 x Cobb 500 FF mixed-sex broilers fed progressive concentrations of digestible lysine (dLys) from 35 to 49 days of age, experiment 1[1]

dLys	Chill Carcass	*P. major*	*P. minor*	Total White Meat	Leg quarters	Wings	Fat pad[2]
(%)	---------- g / bird ----------						%
0.60	2220	473	114	581	709	268	2.81
0.68	2254	486	117	602	712	270	2.81
0.76	2294	503	125	628	725	270	2.66
0.84	2270	506	121	627	715	267	2.49
0.92	2304	514	126	641	725	280	2.37
1.00	2294	514	124	638	729	270	2.15
1.08	2324	528	127	655	729	275	2.22
1.16	2345	537	133	670	740	272	2.18
SEM	78	24	4	27	29	11	0.32
Source of variation	---------- P- value ----------						
Linear	0.000	0.000	0.000	0.000	NB	0.026	0.000
Quadratic	NB[3]	0.005	0.005	0.003	NB	NB	0.001

[1]Values are means of 12 replicate pens per treatment, each with 8 birds (4 males, 4 females) representative of the mean bird weight (± 300 g) per pen at 49 d-of-age.
[2]Values represent % of fat pad surrounding the abdominal viscera and gizzard relative to the respective hot carcass weight.
[3]No significant breakpoints (NB) could be determined for data sets generating linear responses.

Table 4.6. Digestible lysine (dLys) requirements of Cobb 500 x Cobb 500 FF mixed-sex broilers reared from 35 to 49 days of age, experiment 1[1]

Yield parameters	dLys g/bird[2]	dLys %	CP %
BW	21.44	0.95	20.35
BWG	21.22	0.96	16.26
FI	21.58	0.88	19.37
FCR	21.06	0.99	NB
Fat Pad	20.93	1.00	19.56
Chill Carcass	NB[3]	0.79	NB
P. major	22.30	0.94	21.35
P. minor	22.30	0.95	18.90
Leg quarters	NB	NB	NB
Wings	NB	NB	18.26
Total White Meat	22.30	0.95	21.42

[1]Values are estimated based on the linear broken-line model.
[2]Values are based on cumulative digestible lysine intake per bird from 35 to 49 days of age.
[3]No significant breakpoints (NB) could be determined for data sets generating linear responses.

Table 4.7. Live performance of Cobb 500 FF x Hubbard M99 crossbred male broilers fed progressive concentrations of digestible lysine (dLys) from 35 to 49 days of age, experiment 2[1]

dLys	FI	BW	BWG	FCR[2]	Mortality
(%)	---------- g / bird ----------			g / g	(%)
0.62	3041	3540	1175	2.60	1.30
0.71	3008	3608	1252	2.41	1.08
0.80	2908	3638	1300	2.25	1.52
0.89	2871	3701	1355	2.12	0.87
0.97	2801	3697	1339	2.10	1.73
1.06	2750	3701	1338	2.08	2.16
1.15	2769	3706	1365	2.04	2.16
1.24	2719	3717	1347	2.03	1.73
SEM	34	27	20	0.02	0.46
Source of variation	---------- P- value ----------				
Linear	0.000	0.000	0.000	0.000	NA[4]
Quadratic	0.001	0.000	0.000	0.000	NA

[1]Values are means of 12 replicate pens per treatment, each with 42 male birds per pen from 35 to 49 d-of-age.
[2]Values represent feed consumed per bird divided by body weight gain (BWG) corrected for mortality.
[4]Mortality data were not subjected to breakpoint regression analysis, thus, P-values were not applicable (NA).

4.8. Processing yield responses of Cobb 500 FF x Hubbard M99 crossbred male broilers fed progressive concentrations of digestible lysine (dLys) from 35 to 49 days of age, experiment 2[1]

dLys	Chill Carcass	*P. major*	*P. minor*	Total White Meat	Leg quarters	Wings	Fat pad[2]
(%)	---------- g / bird ----------						%
0.62	2583	591	128	719	846	290	2.29
0.71	2609	618	130	748	837	291	2.19
0.80	2642	632	135	765	850	295	1.87
0.89	2707	667	142	801	865	298	1.78
0.97	2691	649	140	788	863	302	1.70
1.06	2691	649	139	786	869	303	1.69
1.15	2736	669	142	811	879	297	1.64
1.24	2745	674	147	821	875	303	1.53
SEM	40	18	4	21	16	8	0.18
Source of variation	---------- P- value ----------						
Linear	0.000	0.000	NB[3]	0.000	0.000	0.000	0.000
Quadratic	0.012	0.008	0.051	0.000	NB	0.002	0.000

[1]Values are means of 12 replicate pens per treatment, each with 8 male birds representative of the mean bird weight (± 300 g) per pen at 49 d-of-age.
[2]Values represent % of fat pad surrounding the abdominal viscera and gizzard relative to the respective hot carcass weight.
[3]No significant breakpoints (NB) could be determined for data sets generating linear responses.

Table 4.9. Digestible lysine requirements of Cobb 500 FF x Hubbard M99 crossbred male broilers reared from 35 to 49 days of age, experiment 2[1]

Yield parameters	dLys g/bird[2]	dLys %	CP %
BW	25.22	0.86	23.53
BWG	24.45	0.86	17.61
FI	28.56	1.07	20.65
FCR	25.61	0.91	18.37
Fat Pad	27.05	0.94	19.60
Chill Carcass	26.05	1.02	18.65
P. major	24.94	0.87	17.94
P. minor	NB[3]	NB	18.19
Leg quarters	30.66	NB	22.18
Wings	26.57	0.94	19.35
Total White Meat	25.28	0.90	18.14

[1]Values are estimated based on the linear broken-line model.
[2]Values are based on cumulative digestible lysine intake per bird from 35 to 49 days of age.
[3]No significant breakpoints (NB) could be determined for data sets generating linear responses.

CHAPTER 5

DIGESTIBLE AMINO ACID REQUIREMENTS OF COBB X COBB 500 FF AND COBB X HUBBARD M99 MALE BROILERS REARED FROM FOURTEEN TO TWENTY-EIGHT DAYS OF AGE UNDER VARYING ENVIRONMENTAL TEMPERATURES[1]

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ABSTRACT

Two trials were conducted to determine a digestible amino acid requirement of male broilers for optimal growth and feed conversion efficiency from 14 to 28 days of age. Experiment one was conducted to determine the optimal digestible lysine (**dLys**) and digestible threonine (**dThr**) requirement for Cobb 500 FF x Hubbard M99 crossbred male chicks while the second experiment evaluated the dLys requirement of male Cobb x Cobb 500 FF broilers reared under thermo-neutral or elevated environmental temperatures. In both experiments, day-of-hatch male chicks were randomly allotted to floor pens at commercial bird density levels and fed the same starter diet from 0 to 14 days of age. On day 14 of age, pens were assigned to one of 11 treatment diets (Experiment 1) or one of 7 treatment diets (Experiment 2). All diets were corn-soy based and isocaloric (3,215 kcal/kg). In Experiment 1, crude protein (**CP**) levels were maintained equal as dThr/dLys ratios increased from 0.42 to 1.03 while in Experiment 2, CP increased as dLys increased incrementally from 0.88 to 1.30%. Additionally, in Experiment 2, two temperature protocols were implemented: recommended (decreasing from 30° C to 23.9°C from day 14 to day 28) and elevated temperature (maintained at 30°C). Using broken-line regression analysis, in Experiment 1, the optimal dietary dLys inclusion level was estimated as 1.02% for body weight (**BW**), and body weight gain (**BWG**), and 1.03% for feed conversion ratio (**FCR**). The optimal dThr level as a percent of diet was estimated to be 0.64 for BW and BWG, and 0.65 for FCR. In Experiment 2, the dietary dLys requirementS for BW, BWG, and FCR for broilers raised in thermo-neutral ambient conditions were estimated as 1.09, 1.10, and 1.19%, respectively. Rearing broilers under elevated temperatures negatively influenced growth and resulted in dietary dLys requirements of 1.12, 1.11, and 1.14% for BW, BWG, and FCR, respectively. Relative to broilers raised under recommended temperature protocols, increasing

the amino acid density of the diet will, to a point, increase the broilers consumption of amino acids thereby improving BW, BWG, and FCR.

KEYWORDS: digestible lysine, digestible threonine, broiler, heat stress

INTRODUCTION

In the past decade, the broiler industry has placed great focus on optimizing breast muscle yield to meet increased consumer demand for white meat. As broiler strains continue to be selected for superior meat yield and lower feed intake, more protein dense diets are necessary to maintain optimal performance (Corzo et al., 2005; Corzo et al., 2010; Kidd et al., 2004). Formulating high protein diets, however, increases feed costs while feeding sub-marginal levels decreases growth performance. In broilers raised to 63 days of age to maximize meat yield, 70% of total feed intake during the bird's life will be consumed between 5 to 9 weeks of age (Cobb-Vantress, 2008a). Thus, as reviewed by Dozier et al. (2008), supplementing diets with higher amino acid levels early in development can be economically advantageous. The cost of providing amino acid dense diets during the starter period (days 0 to 14 of age) can be offset by the lower quantity of diet consumed by young broilers relative to older broilers combined with potential higher margins of return resulting from improved breast meat yield at market time that result from performance enhancement gained in the starter period. Similarly, targeting the grower period, which corresponds to 14 to 28 days of age, may provide analogous results.

While diets containing low dietary protein in general limit muscle synthesis by reducing myofibril size and transcription of myogenic genes (Duclos 2005; Duclos et al., 1993; Rosebrough et al., 1996; Tesseraud et al., 2006; Tesseraud et al., 2003), lysine (**Lys**) in particular, is highly incorporated into the fast-twitch fibers of the *pectoralis major* and *minor* and therefore, more profoundly impacts breast muscle formation relative to other essential amino acids (Munks et al., 1945; Tesseraud et al., 1996). However, interactions between Lys and other amino acids have also been reported to affect breast muscle accretion. To that end, in separate trials Kidd et al. (1997) and Kerr et al. (1999) demonstrated that dietary requirements for

threonine (**Thr**) increased accordingly with higher dietary Lys supplementation in order to maximize white meat yield.

Most broiler production in the United States occurs in regions where humidity is high and daytime summer temperatures often increase beyond the capabilities of broiler house environmental control equipment to maintain broilers within their thermo-neutral zone. Erratic and prolonged exposure to such conditions can result in poor growth performance due to lower feed intake, lower body weight, altered muscle protein deposition, higher mortality, and immunosuppression of broilers (Bottje and Harrison, 1985; Temim et al., 2000b; Yahav et al., 1995; Young 1990). Recognizing the effects of environment upon production, nutritionists attempt to reduce such effects by altering nutrient density with varying results (Temim et al., 2000a). However, most heat stress studies have not concurrently evaluated the digestible lysine (**dLys**) requirement of broilers in a large floor pen setting across thermo-neutral and elevated environmental temperatures.

Therefore, the objectives of the current research were to 1) define the dLys requirement of male broilers derived from a Cobb 500 fast feathering (**FF**) female x Hubbard M99 male cross reared under recommended temperatures from 14 to 28 days of age when dietary digestible threonine (**dThr**) levels exceed requirments, 2) define the dThr requirement of male broilers derived from a Cobb 500 fast feathering (**FF**) female x Hubbard M99 male cross reared under recommended temperatures from 14 to 28 days of age when dietary dLys levels are in excess, and 3) to determine the dLys requirement of male Cobb x Cobb 500 FF broilers reared under thermo-neutral versus elevated environmental conditions from 14 to 28 days of age.

MATERIALS AND METHODS

Bird Husbandry

Two experiments were conducted in a facility previously described (Chapter 4). Preceding the start of the experiment, litter used by 3 previous boiler flocks was top-dressed with fresh pine shavings. The lighting schedule (0 to 28 days of age), temperature settings (0 to 28 days of age), and vaccine protocols were followed as detailed previously (Chapter 4), unless otherwise noted. All animal procedures were approved by the University of Georgia Animal Care and Use Committee, Athens, GA.

Experiment 1.

Prior to placing chicks, 88 pens were assigned to one of 11 dietary grower treatments in a random block design with 8 replicates per treatment, 2 replicate pens per treatment per room in the growing facility. Day of hatch male broilers from a Cobb 500 fast feathering (FF) female x Hubbard M99 male cross were weighed and randomly assigned to the 88 pens (52 birds per pen, 0.072 m^2/bird stocking density).

Experiment 2

Pens and rooms were equipped identically to experiment 1 except that prior to placing chicks, the pens had been reduced in size by half (1.52 m by 1.22 m) and 84 pens were assigned to one of 7 dietary treatments in a random block design. Day of hatch male Cobb 500 x Cobb 500 FF chicks were weighed and allocated into 84 pens (28 chicks per pen, 21 pens per room) at a stocking density of 0.063 m^2/bird. On day 14, chicks were reduced to 25 birds per pen (0.072 m^2/bird, stocking density). From 14 to 28 days, the 4 rooms that the treatments were blocked across were divided into two temperature protocols: 2 rooms were maintained at recommended ambient temperature and 2 rooms were maintained with elevated temperatures. Thus, there were

6 replicates pens (3 replicate pens in each of the 2 rooms at the same temperature) for each of the 7 dietary treatments for each temperature protocol. To achieve the temperature extremes between the rooms, the temperature in 2 of the rooms was reduced from 30° C by 0.56° C per day until 23.9° C was reached (Table 5.1). In contrast, the temperature of the 2 remaining rooms was maintained between 30 and 31.82 °C from 14 to 28 d resulting in an elevated ambient temperature (Table 5.1).

Dietary Treatments

Feed and water were provided ad libitum for the starter (0 to 14 days), and grower (14 to 28 days) periods. The common starter and the grower phase treatment diets were formulated to meet or exceed 1994 NRC requirements (Tables 5.2 and 5.3) except for the amino acid being tested for its requirement in the grower phase. Essential AA ratios were maintained across all diets with the exception of the test AA in each trial. In both experiments, the grower diets were derived from common base and summit mixes. Diet formulations and feed analyses were conducted as described in Chapter 4.

Experiment 1

In order to create the 11 dietary treatments, two base mixes and one summit mix were blended in varying proportions as described in Table 5.4. Base 1 (low dLys, high dThr) supplied 3,120 kcal/kg, 21.42% CP, 0.92% dLys, and 0.95% dThr while base 2 (high dLys, low dThr) supplied 3,120 kcal/kg, 21.56% CP, 1.32% dLys, and 0.55% dThr. The summit mix (high dLys, high dThr) supplied 3,120 kcal/kg, 21.56% CP, 1.32% dLys, and 0.95% dThr. Thus, as the basal and summit mixes were blended, metabolizable energy and CP levels were maintained across the experimental diets as dLys:dThr levels varied between titration points (Table 5.4). Dietary treatments 1 through 6 (Table 5.4) were designed to allow the determination of the dLys

requirement while maintaining a constant high dThr level across the titrated dLys diets. In contrast, dietary treatments 6 through 11 were designed to allow the determination of the dThr requirement while maintaining a constant high dLys level across the titrated dThr diets.

Experiment 2

Diets for the grower phase of experiment 2 were created from 1 base diet and 1 summit diet. The base diet supplied 3,120 kcal/kg, 17.40% CP, and 0.88% dLys while the summit diet supplied 3,120 kcal/kg, 23.99% CP, and 1.30% dLys (Table 5.3). Treatment 1 consisted of 100% base diet and treatment diet 7 consisted of 100% summit diet. The five intermediate titration points were created by blending the base and summit diets in varying proportions as described in Table 5.5. Consequently, the diets were isocaloric and met minimum essential AA ratios across all treatments; however, dietary CP levels varied as dLys levels increased in 0.07% increments between the 7 dietary treatments.

Measurements

For each room, humidity, temperature, water consumption, and pen mortality were recorded daily. In addition, for experiment 2, automated Hobo data loggers (Pocasset, MA) were placed in rooms (5 loggers per room) to measure temperature and humidity in 5 min intervals. On days 14 and 28, chicks and feed were weighed and recorded in order to determine body weight (**BW**), feed intake (**FI**), body weight gain (**BWG**), and the feed conversion ratios (**FCR**) for each pen. All grow-out data were corrected for BW and day of individual bird mortality.

Statistical Analyses

ANOVA using the GLM was conducted to evaluate the completely randomized block design and significant room effects were not detected. On day 14, ANOVA using the GLM was utilized to determine that BW did not differ significantly between treatment pens prior to

initiating feeding with the experimental diets. ANOVA calculations were done with Minitab (Release 13, State College, PA) whereas live performance data were subjected to broken-line regression analyses to determine dLys and dThr requirements (Vedenov and Pesti, 2007). Results were considered significant when $P < 0.05$.

RESULTS

Experiment 1

A significant positive response to increasing dietary dLys levels was observed for BW, BWG and FCR (Table 5.6). Using the linear broken-line model, the dLys requirement from 14 to 28 days of age for male broilers from a Cobb 500 FF female x Hubbard M99 male cross as a percent of diet was 1.2, 1.02 and 1.03 for BW, BWG, and FCR, respectively (Table 5.7). Similarly, a significant positive response to increasing dietary dThr levels was observed for BW, BWG and FCR (Table 5.6). Using the linear broken-line model, the dThr requirement from 14 to 28 days of age as a percent of diet was 0.64, 0.64 and 0.65 for BW, BWG, and FCR, respectively for male broilers from a Cobb 500 FF female x Hubbard M99 male cross (Table 5.7).

Experiment 2

For every dietary treatment, Cobb x Cobb 500 FF broilers reared from 14 to 28 days of age under recommended, thermo-neutral environmental temperatures had higher FI, BW, and BWG as well as lower FCR than equivalent broilers reared from 14 to 28 days at an ambient temperature of 30 °C (Table 5.8). Improvements in BW, BWG, and FCR were realized with increasing dietary levels of dLys in both the broilers raised under a normal temperature and those raised under elevated temperature protocol (Table 5.8). Using the linear broken-line model, the

dLys requirement from 14 to 28 days of age as a percent of diet was 1.09, 1.10, and 1.19 for BW, BWG, and FCR, respectively for male broilers from the Cobb x Cobb 500 FF cross reared following a normal temperature protocol (Table 5.9). On the other hand the same broilers reared under elevated ambient temperatures had a dLys requirement from 14 to 28 days of age as a percent of diet of 1.12, 1.11 1.26 and 1.14 for BW, BWG, FI, and FCR, respectively (Table 5.9).

DISCUSSION

In experiment 1, the dLys and dThr requirement of male Cobb 500 FF female x Hubbard M99 male broilers from 14 to 28 days of age were determined as a percent of the diet as 1.023 and 0.643 based on averaging the requirements for BW, BWG, and FI. The dLys requirement was considerably lower than the dLys requirement of 1.127% (averaging the requirements for BW, BWG, and FCR) determined for the same time period in male Cobb x Cobb 500 FF broilers. The determined dLys and dThr requirements were also lower based on similar requirements published for broilers during similar age periods. Dozier et al. (2009), determined the dLys requirement of male Ross x Ross TP16 broilers from 14 to 28 days of age as a percent of diet to be 1.09 and 1.15 for BWG and FCR, respectively. Another experiment utilizing 14 to 28 d-old male progeny from an Avian female x Ross male cross, resulted in estimated dLys requirement of 0.99% for BWG (Labadan et al., 2001). Additionally, in a synopsis of trials conducted with several broiler strains across varying phases of grow-out, Rostagno et al. (2007) reanalyzed dLys requirements concluding that for optimal FCR in Cobb male broiler chicks, the dLys requirement is 1.16% for 10 to 21 days of age and 1.04% from 22 to 35 days of age. Mehri et al. (2012) evaluated AA interactions between Met, Lys, and Thr in 3 to 16 d old Ross x Ross 308 male broilers and suggested that optimal dietary dLys, dMet, and dThr values were 1.12,

0.54, and 0.78%, respectively, for BWG and 1.13, 0.53, and 0.75%, respectively, for FCR. Rosa et al. (2001) estimated that the dThr requirements were 0.69 and 0.68% for BWG and FCR, respectively, in a high yield strain of broilers during the starter period (0 to 18 d), while Samadi and Liebert (2006) determined that the dThr requirement for male Cobb 500 chicks was 0.78% from 10 to 25 days of age.

In chapter 4 of this dissertation, the dLys requirement for male broilers from the Cobb 500 FF female x Hubbard M99 male cross was determined from 35 to 49 days of age and again the dLys requirement was lower than expected based on current and previous research. It appears the Cobb 500 FF female x Hubbard M99 male cross produces a very efficient offspring with regard to dLys and dThr requirements. This cross has gained popularity in recent years as the number of broiler progeny derived from the Cobb x Hubbard cross has increased (Agristats, 2010).

Kidd, et al. (1997) and Kerr, et al. (1999) reported that dietary requirements for Thr increased when dietary levels of Lys were elevated to increase breast meat yields. In the current research, a very high dietary level (1.32%) of dLys was used in the diets for the dThr titration curve with the expectation that this would result in an elevated dThr requirement relative to other studies. Similarly, a high level of dThr was used in the diets for the dLys titration curve with the expectation that it might increase the dLys requirement based on the reported Lys x Thr interaction. As indicated earlier, our determined dLys and dThr requirements were very low and suggest that a significant Lys x Thr interaction might not exist in broilers from 14 to 28 day of age. This would agree with the recent report by Everett et al. (2010) that no significant interactions between Lys and Thr were detected in 14 to 28 day old Ross x Ross TP16 male

broilers. However, Everett et al. (2010) did determine that a significant interaction between Lys and Thr occurred for BWG in broilers from 28 to 42 days of age.

Aside from the effects of genetic strain, sex, age, and AA interactions on determining dThr requirement patterns in broilers, the discrepancies found across studies may be partially explained by differences in enteric pathogen challenges experienced by broilers during grow-out. Threonine is highly incorporated into mucins produced by intestinal epithelial cells and therefore is required for the maintenance of the mucosal gut barrier (Bertolo et al., 1998; Schaart et al., 2005). Increasing the dietary Thr:Lys ratio from 0.63 to 0.68 during challenges with enteric pathogens has been shown to improve growth performance in broiler chicks in relation to non-infected birds (Star et al., 2012). Thus, differences in dThr requirements might result from even subclinical differences in enteric pathogen challenges between different experiments and may partially account for contrasting dThr requirements reported by different researchers.

In experiment 2, based on broken-line regression analyses, the dLys requirements for Cobb x Cobb 500 FF male broilers reared under recommended environmental temperatures from 14 to 28 days of age were estimated to be 1.12% for BW, 1.10% for BWG, and 1.19% for FCR. The higher dLys levels required for optimal FCR relative to BWG is consistent with previously published data (Han and Baker, 1993; Leclercq, 1998; Mack et al., 1999; Baker et al., 2002; Dozier et al., 2009). In the current research, although BW and BWG plateaued as dietary dLys levels continued to increase, feed intake continued to decrease such that a broken-line regression analysis requirement could not be calculated. However, even though feed intake is still decreasing in a linear manner without a breakpoint, the plateau in BWG is enough to ultimately lead to a decrease in FCR such that the broken line regression software assigns a breakpoint that is inflated.

Rearing broilers under elevated environmental temperatures induces physiological and metabolic changes that allow birds to cope with heat stress, but which detrimentally impact growth performance parameters (Yahav et al., 1995; Temim et al., 2000a; Temim et al., 2000b; Tankson et al., 2001; Dridi et al., 2008). In experiment 2, one half of the birds were exposed to 30 °C constantly without reductions of ambient heat to simulate diurnal cycles where morning and evening temperatures are typically cooler. Relative to broilers confronted with constant heat-stress, birds reared under cyclic heat-stress conditions adapt and achieve superior growth (Deaton et al., 1984) by feeding most actively early and late in the day, thereby reducing metabolic heat expenditure during hours of maximum heat stress. To the contrary, in experiment 2 of the current research, persistent heat-stress conditions exacerbated feed intake suppression as the broilers minimized consumption throughout the day. Subsequently, though all birds were provisioned with the same treatment diets, heat-stressed chicks exhibited lower BW and BWG but higher dLys requirements to optimize BWG. For FCR, on the other hand, the dLys requirement of broilers reared under elevated environmental temperatures was 1.14%, which equated to a 0.05% requirement reduction in comparison to the FCR of the broilers raised in the ideal environment. Similar differences in FCR have been observed by Han and Baker (1993) and are likely an outcome of smaller body size of heat-stressed chicks and proportionately lower maintenance energy cost.

Modifying diets to increase AA density has been shown to alleviate the affects of low feed intake by increasing the broilers' net consumption of AA and thereby improving performance parameters during exposure to high ambient temperatures (McNaughton and Reece, 1984; Corzo et al., 2003; Gonzalez-Esquerra and Leeson, 2006). This was seen in the current research as well.

In summary, the current research indicates that the male offspring resulting from the Cobb 500 FF female x Hubbard M99 male have lower dLys and dThr requirements than other modern broiler crosses. For heat stressed broilers increasing the amino acid density of the diet will, to a point, increase the broilers consumption of amino acids thereby improving BW, BWG, and FCR.

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Table 5.1. Temperature protocol for thermo-neutral (TN) and high ambient temperature (30°C) rooms, experiment 2

	Intended		Actual[1]	
Day	TN[2]	30°C	TN	30°C
14	30.00	30.00	29.96	30.43
15	29.44	30.00	29.21	30.32
16	28.89	30.00	28.79	30.46
17	28.33	30.00	28.34	30.32
18	27.78	30.00	27.42	29.73
19	27.22	30.00	27.30	30.41
20	26.67	30.00	27.01	30.03
21	26.11	30.00	26.93	30.40
22	25.56	30.00	27.10	31.43
23	25.00	30.00	26.89	31.51
24	24.44	30.00	26.59	31.18
25	23.89	30.00	25.95	31.04
26	23.33	30.00	26.73	31.82
27	22.78	30.00	25.79	31.38
28	22.22	30.00	24.95	30.88

[1]Values are based on average temperatures recorded by automated devices every 5 minutes for a 24 hour cycle.
[2]Based on breeder guidelines (Cobb-Vantress Inc., 2008a; 2008b)and facility standard operating procedures.

Table 5.2. Composition of diets for the starter[1] phase, experiments 1 and 2

Ingredient	Experiment 1	Experiment 2
	---------- (%) ----------	
Corn	52.331	57.843
Soybean meal, 48.0%	33.761	33.112
Meat and bone meal	4.664	4.223
Poultry Oil	1.892	2.238
Corn DDGS	5.000	-
Limestone	1.072	1.024
Sodium-Carbonate	0.532	0.620
DL-Met	0.320	0.406
L-Lys·HCl	0.152	0.187
L-Thr	0.050	0.077
L-Val	-	0.031
Phytase[2]	0.020	0.020
Trace mineral mix[3]	0.062	0.063
Vitamin mix[4]	0.025	0.025
Monteban-45	0.063	0.063
Choline chloride 60%	0.020	0.033
BMD-50	0.036	0.036

[1]Starter diet fed from 0 to 14 days of age (crumble diet).
[2]Quantum 2500 XT (AB Vista, NC)
[3]Trace mineral premix provides the following in milligrams per kilogram of diet: manganese, 176; zinc, 176; iron, 64; copper, 8.8; iodine, 2.8; selenium, 0.5.
[4]Vitamin premix provides the following per kilogram of diet: vitamin A, 7,000 IU; vitamin D_3, 2,500 IU; vitamin E, 19 IU; vitamin K, 1.3 mg; vitamin B_1, 1.6 mg; vitamin B_2, 6.3 mg; vitamin B_6, 2.4 mg; vitamin B_{12}, 0.01 mg; niacin, 40 mg; pantothenic acid, 11 mg; folic acid, 0.7 mg; biotin, 0.08 mg.

Table 5.3. Composition of the basal diets for the grower[1] phase, experiments 1 and 2

Ingredient	Experiment 1			Experiment 2	
	Base 1 Low Lys High Thr	Base 2 High Lys Low Thr	Summit High Lys High Thr	Base Low Lys	Summit High Lys
	---------- (%) ----------				
Corn	55.29	55.29	55.29	70.53	51.52
Soybean meal, 48.0 % CP	13.79	13.79	13.79	21.40	37.12
Peanut Meal	16.92	16.92	16.92	-	-
Meat and bone meal	2.91	2.91	2.91	3.49	3.49
DDGS	5.00	5.00	5.00	-	-
Poultry Oil	2.14	2.07	1.93	2.13	5.22
Salt	-	-	-	0.22	0.23
Limestone	1.091	1.091	1.091	1.008	1.037
Defluorinated phosphorus	-	-	-	0.132	-
DL-Met	0.533	0.533	0.533	0.244	0.448
L-Lys·HCl	0.267	0.775	0.775	0.114	0.143
L-Thr	0.410	-	0.410	0.031	0.089
L- Trp	0.048	0.048	0.048	-	-
L-Val	0.272	0.272	0.272	-	0.042
L-Iso	0.252	0.252	0.252	-	-
Gly	0.295	0.257	-	-	-
Coban 90	0.045	0.045	0.045	0.045	0.045
BMD 50	0.036	0.036	0.036	0.036	0.036
Phytase[2]	0.020	0.020	0.020	0.020	0.020
Trace mineral mix[3]	0.063	0.063	0.063	0.063	0.063
Vitamin mix[4]	0.025	0.025	0.025	0.025	0.025
Sodium-Carbonate	0.527	0.527	0.527	0.438	0.451
Choline Chloride 60%	0.064	0.064	0.064	0.079	0.025
Sulkaflock	-	0.015	-	-	-
ME, kcal/kg	3120	3120	3120	3120	3120
CP, %	21.42	21.56	21.56	17.40	23.99
Analyzed CP, %	22.48	22.06	22.40	15.45	23.66
dLys, 100 %	0.92	1.32	1.32	0.88	1.30
dThr:Lys	103.00	42.00	72.00	68.00	68.00
dTSAA:Lys	109.00	76.00	76.00	78.00	78.00
dArg:Lys	151.00	105.00	105.00	120.99	118.03
dIle:Lys	96.00	67.00	67.00	70.74	69.00
dVal:Lys	111.00	77.00	77.00	80.17	78.00
dTrp:Lys	23.00	16.00	16.00	19.00	19.46
dGly:dLys	144.0	98.00	79.00	101.95	89.81

[1]Grower diet fed from 7 to 21 days of age (pelleted diet)

[2]Quantum 2500 XT (AB Vista, NC)
[3]Trace mineral premix provides the following in milligrams per kilogram of diet: manganese, 176; zinc, 176; iron, 64; copper, 8.8; iodine, 2.8; selenium, 0.5.
[4]Vitamin premix provides the following per kilogram of diet: vitamin A, 7,000 IU; vitamin D_3, 2,500 IU; vitamin E, 19 IU; vitamin K, 1.3 mg; vitamin B_1, 1.6 mg; vitamin B_2, 6.3 mg; vitamin B_6, 2.4 mg; vitamin B_{12}, 0.01 mg; niacin, 40 mg; pantothenic acid, 11 mg; folic acid, 0.7 mg; biotin, 0.08 mg.

Table 5.4. Blending of grower diets provided to broilers from 14 to 28 days, experiment 1

Treatment	dLys	dThr	dThr/Lys	Base 1	Base 2	Summit
	(%)	(%)		---------- (%) ----------		
1	0.92	0.95	1.03	100	-	-
2	1.00	0.95	0.95	80	-	20
3	1.08	0.95	0.88	60	-	40
4	1.16	0.95	0.82	40	-	60
5	1.24	0.95	0.77	20	-	80
6	1.32	0.95	0.72	-	-	100
7	1.32	0.55	0.42	-	100	-
8	1.32	0.63	0.48	-	80	20
9	1.32	0.71	0.54	-	60	40
10	1.32	0.79	0.60	-	40	60
11	1.32	0.87	0.66	-	20	80

Table 5.5. Blending of grower diets provided to broilers from 14 to 28 days, experiment 2

Treatment	dLys	CP	Base Diet	Summit Diet
	---------- (%) ----------			
Trt 1 (Base Diet)	0.88	17.40	100	-
Trt 2	0.95	18.50	83.33	16.67
Trt 3	1.02	19.60	66.67	33.33
Trt 4	1.09	20.70	50.00	50.00
Trt 5	1.16	21.79	33.33	66.67
Trt 6	1.23	22.89	16.97	83.33
Trt 7 (Summit Diet)	1.30	23.99	-	100.00

Table 5.6. Live performance of Cobb 500 FF x Hubbard M99 male broilers fed progressive concentrations of digestible lysine or threonine from 14 to 28 d-of-age[1], experiment 1

dLys	dThr	FI	BW	BWG	FCR[2]	Mortality
(%)	(%)	---------- (g / bird) ----------			(g / g)	(%)
0.92	0.95	1553	1480	1064	1.456	1.302
1.00	0.95	1591	1557	1137	1.394	1.042
1.08	0.95	1605	1580	1155	1.388	1.563
1.16	0.95	1595	1577	1162	1.362	1.042
1.24	0.95	1584	1580	1159	1.358	1.042
1.32	0.95	1603	1582	1161	1.363	1.302
1.32	0.55	1523	1462	1043	1.462	0.781
1.32	0.63	1585	1562	1146	1.381	0.521
1.32	0.71	1572	1577	1156	1.364	1.563
1.32	0.79	1558	1552	1135	1.364	0.521
1.32	0.87	1591	1582	1161	1.368	0.781
SEM		19	17	14	0.01	0.794
Overall treatment means		---------- P- value ----------				
Linear broken-line						
dLys		NB[3]	0.000	0.000	0.000	NA[4]
dThr		NB	0.000	0.000	0.000	NA
Quadratic broken-line						
dLys		0.001	0.000	0.000	0.000	NA
dThr		NB	0.000	0.000	0.000	NA

[1]Values are means of 8 replicate pens per treatment, each with 50 broiler males per pen from 14 to 28 d-of-age.
[2]Values represent feed consumed per bird divided by body weight gain (BWG) corrected for mortality.
[3]No significant breakpoints (NB) could be determined for data sets generating linear responses.
[4]Mortality data were not subjected to breakpoint regression analysis thus, P-values were not applicable (NA).

Table 5.7. Dietary digestible lysine and threonine requirements of Cobb 500 FF x Hubbard M99 male broilers from 14 to 28 d-of-age based on broken-line regression[1], experiment 1

	dLys	dThr
Growth parameter	---------- % ----------	
BW	1.02	0.64
BWG	1.02	0.64
FI	NB[2]	NB
FCR	1.03	0.65

[1]Values are means of 8 replicate pens per treatment, each with 50 broiler males per pen from 14 to 28 d-of-age.
[2]No significant breakpoints (NB) could be determined for data sets generating linear responses.

Table 5.8. Live performance of Cobb x Cobb 500 FF male broilers fed progressive concentrations of digestible lysine and reared under thermo-neutral (TN) or elevated ambient temperatures (30°C) from 14 to 28 d-of-age[1], experiment 2

	FI		BW		BWG		FCR[2]		Mortality	
dLys	TN[3]	30 °C[4]	TN	30 °C	TN	30 °C	TN	30 °C	TN	30 °C
(%)	(g / bird)						(g / g)		(%)	
0.88	1582[a]	1328[b]	1423	1199	1048	817	1.510	1.629	0.00	0.00
0.95	1575[a]	1356[b]	1468	1266	1086	881	1.461	1.545	0.67	1.33
1.02	1510[c]	1332[b]	1454	1278	1078	895	1.410	1.499	2.00	0.00
1.09	1522	1310	1499	1293	1124	920	1.341	1.440	0.00	0.67
1.16	1474	1306	1507	1305	1126	928	1.311	1.410	0.00	0.00
1.23	1426	1299	1476	1326	1108	944	1.289	1.381	0.00	0.67
1.30	1415	1295	1496	1321	1126	937	1.270	1.387	0.67	0.00
SEM	17	16	13	15	13	15	0.005	0.008	0.273	0.269
Overall treatment means	P- value									
Linear	NB[5]	0.001	0.000	0.000	0.000	0.000	0.000	0.000	NA[6]	NA
Quadratic	NB	NB	0.001	0.000	0.001	0.000	0.000	0.000	NA	NA

[1]Values are means of 12 replicate pens per treatment, each with 25 broiler males per pen from 14 to 28 d-of-age.
[2]Values represent feed consumed per bird divided by body weight gain (BWG) corrected for mortality.
[3]To achieve thermo-neutral (TN) conditions, ambient temperature was decreased by 0.56° C per day until 23.9 °C was reached.
[4]Elevated ambient temperatures were constantly maintained at 30°C, or above.
[5]No significant breakpoints (NB) could be determined for data sets generating linear responses
[6]Mortality data were not subjected to breakpoint regression analysis thus, P-values were not applicable (NA).
[a-c]Means without a common letter differ, ($P < 0.005$).

Table 5.9. Dietary digestible lysine requirements of Cobb x Cobb 500 FF male broilers reared under thermo-neutral (TN) or elevated ambient temperatures (30°C) from 14 to 28 d-of-age[1], experiment 2

	TN[1]	30 °C[2]
Growth parameter	dLys, %	
BW	1.09	1.12
BWG	1.10	1.11
FI	NB[3]	1.26
FCR	1.19	1.14

[1]To achieve thermoneutral (TN) conditions, ambient temperature was decreased by 0.56° C per day until 23.9° C was reached.
[2]Elevated ambient temperatures were constantly maintained at 30°C, or above.
[3]No significant breakpoints (NB) could be determined for data sets generating linear responses.

CHAPTER 6

CHARACTERIZATION OF AVIAN GHRELIN O-ACYLTRANSFERASE (GOAT) TISSUE mRNA DISTRIBUTION IN THE BROILER CHICKEN[1]

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ABSTRACT

In vertebrates, ghrelin is a highly conserved protein hormone involved in a wide range of regulatory pathways effecting energy metabolism and expenditure. Ghrelin is produced by the acylation of the 3rd amino acid of unacylated ghrelin by the enzyme ghrelin O-acyltransferase (**GOAT**). Previous research in our laboratory indicated that plasma ghrelin levels were elevated in fasted broiler breeder hens, therefore, the goal of the current research was to characterize GOAT mRNA expression in various broiler tissues and to determine if GOAT mRNA expression increased in fasted BB hens. Using mouse and human GOAT mRNA sequences as search queries, a putative chicken GOAT mRNA sequence was identified from the chicken genome. RT-PCR primers were designed based on the identified sequence and PCR products were amplified from broiler proventriculus cDNA and sequenced to verify the putative GOAT sequence. Taqman MGB probes and primers for detecting GOAT and GAPDH (endogenous control) were designed for real time RT-PCR analyses of extracted total RNA. Total RNA was isolated from various organ tissues of young broilers as well as the proventriculus and ovarian tissues of mature broiler breeder hens that had either been fed or fasted for 72 hours. GOAT mRNA expression was most abundant in the proventriculus and was significantly greater in the proventriculus of fasted broiler breeder hens relative to fed hens. Low levels of GOAT were detected in small and large intestine as well as granulosa tissue isolated from small preovulatory follicles. GOAT mRNA was not detected in the liver, adrenal glands, kidneys or theca tissue. Granulosa cell GOAT mRNA expression decreased with follicular maturation. Collectively, the current research parallels previously published mammalian data where GOAT expression is most prominent in glandular gastric tissue and increases under caloric restriction.

KEYWORDS: proventriculus, intestine, fasted broiler, granulosa

INTRODUCTION

Ghrelin is a highly conserved, 28-amino acid, peptide hormone produced predominantly by the stomach. Currently, two forms of ghrelin, unacylated ghrelin and acylated ghrelin, are known (Date et al., 2000; Hosoda et al., 2000; Hosoda et al., 2003). Acylation of the third N-terminal amino acid, serine 3 (**Ser3**), of ghrelin with a fatty acid (n-octanoic acid) moiety is essential for binding and activation of ghrelin's cognate receptor, growth hormone secretagauge receptor (GHSR) (Gutierrez et al., 2008; Yang et al., 2008), through which, ghrelin elicits the release of growth hormone. In addition to stimulating growth hormone release, ghrelin is also involved in the regulation of feeding behavior and energy homeostasis. In rodents, fasting induces gastric ghrelin mRNA expression and increases secretion of the ghrelin peptide into blood circulation while re-feeding decreases both gastric mRNA and plasma total ghrelin content (Toshinai et al., 2001). Moreover, infusion of ghrelin, either intravenously or peripherally, has been reported to stimulate feeding behavior with chronic administration leading to weight gain (Tschop et al., 2000; Kamegai et al., 2001; Wren et al., 2002; Volkoff et al., 2005; Valassi et al., 2008). Thus, in mammals, ghrelin is believed to signal the lack of caloric intake from the stomach to the CNS where feeding behavior is initiated when high ghrelin levels indicate a lack of nutrients.

Avian ghrelin was first isolated in 2002 from chicken proventriculus, the glandular stomach in birds (Kaiya et al., 2002). The 26-amino acid peptide is 54% homologous to human and rat ghrelin and is highly conserved among investigated avian species (Kaiya et al., 2002; Kaiya et al., 2007; Kaiya et al., 2008). Similar to mammalian species, studies conducted on quail and chickens indicate that caloric restriction in birds leads to higher plasma ghrelin concentrations, which decrease upon re-feeding (Shousha et al., 2005; Kaiya et al., 2007).

During fasting, ghrelin mRNA expression levels increase in the proventriculus and liver, but are not restored to control levels until several hours post feeding even though plasma levels of ghrelin decrease quickly upon re-feeding (Chen et al., 2007; Kaiya et al., 2007; Richards and McMurtry, 2010).

Due to the correlation between feed deprivation and ghrelin synthesis, ghrelin is believed to signal a negative energy balance state between the peripheral tissues and the CNS in avian species. However, the effect of ghrelin on feeding behavior in birds may contrast to that found in mammals. Since the discovery of ghrelin in avian species, there has been only one report of peripherally injected ghrelin stimulating feeding behavior in birds and that occurred in adult quail (Shousha et al., 2005). Intracerebroventricular injection of ghrelin into broiler chicks or peripheral injection of ghrelin into Leghorn chicks inhibited (Furuse et al., 2001; Saito et al., 2002; Kaiya et al., 2007) or had no effect on feed intake (Kaiya et al., 2007). Feed intake aside, avian ghrelin biology parallels mammalian ghrelin biology in that the glandular stomach is the predominant site for ghrelin production and secretion into blood circulation (Kaiya et al., 2002; Kaiya et al., 2008; Kaiya et al., 2009), gastric proghrelin mRNA expression increases during fasting (Yamato et al., 2005; Richards et al., 2006; Kaiya et al., 2007), and availability of octanoic acid from the diet increase the ratio of plasma acylated ghrelin to total ghrelin (Kirchner et al., 2009).

In 2008, the enzyme responsible for ghrelin acylation was identified as ghrelin O-acyltransferase (**GOAT**), a member of the membrane bound O-acyltransferase (MBOAT) family (Gutierrez et al., 2008; Yang et al., 2008). Similarly to its substrate, ghrelin, GOAT and its catalytic properties are also highly conserved across species (Gutierrez et al., 2008). In mammals, GOAT mRNA has been detected in various tissues throughout the body with the

highest level detected in the stomach (Gutierrez et al., 2008; Yang et al., 2008). Just as ghrelin synthesis is stimulated by caloric restriction, GOAT mRNA levels have been reported to increase during fasting (Gonzalez et al., 2008).

In previous research from our laboratory, plasma ghrelin levels were reported to increase in broiler breeder hens that were fasted (Freeman, 2008). Additionally, the mRNA for the ghrelin receptor was detected in the theca and granulosa cells from hierarchical and nonhierarchical follicles and the mRNA expression of the ghrelin receptor was down-regulated by FSH and LH in cultured granulosa cells (Freeman, 2008). Therefore, the goal of the current research was to confirm the existence of and determine the tissue expression pattern of avian GOAT mRNA and to determine if feed deprivation alters GOAT mRNA expression.

MATERIALS AND METHODS

Experiment 1

The purpose of this experiment was to determine if GOAT mRNA could be detected in selected tissues of growing broilers. Cobb 500 X Cobb 500 fast feathering female broilers, which had been hatched and vent sexed at the University of Georgia Poultry Research Center, were reared from day of hatch to 35 days of age in floor pens. They were fed standard corn/soybean based starter, grower, and withdrawal broiler diets. The floor pens were in an environmentally controlled facility. Ambient temperature was set to 34 °C on day 1 and decreased by 0.28 °C until 24 °C was reached and then maintained. A continuous lighting program was implemented with a light intensity of 20 lux for 24 hours (0 to 4 days of age), 20 lux for 20 hours (5 to 7 days of age), 10 lux for 16 hours (8 to 14 days of age), and 2 lux for 16 hours (15 to 35 days of age). Birds were provided with water and feed ad libitum. All animal

procedures were approved by the University of Georgia Animal Care and Use Committee, Athens, GA.

At 5 weeks-of-age, birds were selected randomly and removed from feed for 2 hours prior to being killed via cervical dislocation for tissue collection. Approximately 300 mg of proventriculus, duodenum, ileum, large intestine, liver, kidney, and adrenal gland tissue was collected from 8 individual birds. Immediately after collection, each tissue was placed in 3 mL of guanidinium isothiocyanate solution and homogenized for 30 seconds with a PowerGen 700 tissue disrupter (Fisher Scientific, Pittsburg, PA). Individual homegenized tissue solutions were frozen and stored at -80ºC for future RNA extraction.

Experiment 2

To determine if GOAT mRNA expression varied in the proximal and distal portions of the proventriculus, 12, five week old male and female Cobb 500 x Cobb 500 fast feathering broilers were obtained from a commercial farm where standard industry feeding and environmental control regimens were followed. Birds did not have access to feed for approximately 4 hours prior to being killed for tissue collection. From each bird, 300 mg tissue samples from the proximal and distal ends of the glandular proventriculus were obtained for RNA extraction. Once obtained the samples were processed as described for experiment 1.

Experiment 3

This experiment was completed to determine if the mRNA for GOAT was expressed in the hierarchical or prehierarchical follicles of broiler breeder hens and if its expression was influenced by fasting. Cobb 500 broiler breeder hens that were between 45 and 55 weeks of age were utilized for this experiment. The birds were reared using a skip a day feed restriction program as previously described in Spradley et al. (2008). At 21 weeks of age the pullets were

placed in individual cages and were photostimulated to initiate reproduction with a lighting program that provided 14L:8D (lights on at 06:30 hours) per day. The hens were given free access to water and were fed a standard broiler breeder layer diet at 08:00 hours. The daily amount of feed provided to the hens was determined using the guidelines of the primary breeder (Cobb-Vantress 2005a, Cobb-Vantress 2005b) based on the weekly body weight measurements and egg production rate of the hens. Eggs were collected twice daily and individual hen egg production was recorded. All animal procedures were approved by the University of Georgia Animal Care and Use Committee, Athens, GA.

Four hens in mid-laying sequence were divided into 2 treatment groups. One treatment group continued to receive the daily allotment of feed while the other treatment group was fasted for 72 hours. At 72 hours, all 4 hens were euthanized for tissue collection. A 200 mg portion of the glandular proventriculus for each hen was collected separately while the pituitary glands were combined from the 2 hens in each treatment to account for low tissue availability (n =6 for proventriculus, n = 3 for pituitary for each feeding state). In addition, the F_1, F_2, F_3, F_4, small yellow (SY; 5-10 mm in diameter), and large white (LW; 2-5 mm in diameter) follicles from each hen were collected for subsequent theca and granulosa cell layer separation for each follicle size. The theca and granulosa cell layers from follicles F_1 through F_4 were manually separated (Huang and Nalbandov, 1997) while for SY and LW follicles the theca and granulosa cell layers were separated enzymatically (Davis et al., 2000). Individual theca and granulosa samples from the 2 birds within each feeding state were combined. This collection procedure was repeated 2 more times to give 3 total replications for each treatment (n = 3).

RNA Extraction

Total RNA was extracted from tissue samples using the guanidinium isothiocyanate-phenol-chloroform method (Chomczynski and Sacchi, 1987). The integrity of each RNA sample was assessed by the presence of intact bands for 28S and 18S rRNA on a 1.5% agarose gel stained with ethidium bromide. RNA samples were stored at -80ºC. Based on the quality of the RNA, the best 6 replicate samples were chosen out of the 8 and 12 replicate samples for experiments 1 and 2, respectively, for the determination of the mRNA expression of GOAT.

RT-PCR

In 2008, Yang et al., cloned mouse GOAT and searched the chicken genome with their sequence and identified a putative GOAT sequence (GenBank NP_001186218). In the current research, PCR primers for chicken GOAT were designed based on this putative sequence using Primer BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast). Three primer sets (Table 6.1) that were designed to collectively obtain the full length mRNA sequence of GOAT were synthesized (University of Georgia Genomics Facility, Athens, GA).

Total RNA isolated from the proventriculus of a broiler was converted to cDNA using the TaqMan Reverse Transcription Kit (Applied Biosystems, Foster City, CA) following the manufacturer's protocol. Subsequently, 20 ng of cDNA was used for PCR using a Takara hot Start PCR Kit (Clontech, Mountain View, CA). PCR was conducted as previously described Davis and Johnson (1998) with annealing temperatures set to 65°C for higher GOAT primer binding specificity.

To produce a sufficient quantity of each PCR product obtained from each of the primer pairs for sequencing, each one was cloned into the pCR®2.1 vector using the TA Cloning kit (Invitrogen, Carlsbad, CA) and expressed in DH5-α competent cells according to manufacturer's

instructions. The expected DNA sequences were verified by the University of Georgia, Georgia Genomics Facility, Athens, GA.

Real Time RT-PCR

Taqman minor groove-binding probes and primers (Table 6.2) for Real Time RT-PCR were designed using Primer Express Software (Version 2.0, Applied Biosystems) and were synthesized by Applied Biosystems. The primer and probe set for GOAT were designed based on sequence information obtained from the RT-PCR products while the endogenous control glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primer and probe set was based on a previously published sequence (GenBank accession M11213). Probes for both GOAT and GAPDH were labeled at the 5' end with reporter dye FAM (6-carboxyfluorescein) and with 3' quencher dye TAMRA (6-carboxy-N,N,N',N'-tetramethylrhodamine). Primer and probe sets were validated for real-time PCR following the manufacturer's protocol (Applied Biosystems, Foster City, CA) to determine optimal primer/probe concentrations and amplification efficiency.

Extracted RNA samples were DNase treated (TURBO DNA-free kit, Ambion, Austin, TX) to remove genomic DNA contamination and subjected to two-step real time PCR. Reverse-transcription cDNA synthesis reactions were completed utilizing the TaqMan Reverse Transcription Kit (Applied Biosystems, Foster City, CA). For real-time PCR, 25µL reactions were prepared with 200ng cDNA, 1x TaqMan Universal PCR Master Mix (Applied Biosystems), 900nM of either GOAT or GAPDH primer pairs, and 25nM of probe. The reactions for each tissue sample were completed in duplicate for both GOAT and GAPDH detection. Reactions were completed in an ABI 7500 Thermocycler (Applied Biosystems) with the cycling program set for 95°C for 10 min followed by 40 cycles each consisting of 95°C for 15 seconds and 60°C for 1 minute. Sequence Detection software (version 1.2.2, Applied Biosystems) was used to

determine the cycle threshold (C_T) for each reaction. Relative quantification was completed using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). Briefly, the GOAT Ct was determined for each sample and then normalized to the GAPDH Ct from the same sample (GAPDH Ct subtracted from the GOAT Ct yielded the ΔCt). After all the ΔCt values were obtained for a replicate experiment, the ΔCt values were all compared relative to the sample with the highest mRNA expression of GOAT using the $2^{-\Delta\Delta Ct}$ method. Thus, the GOAT mRNA expression data for each individual sample is expressed as the fold-difference relative to sample with the highest GOAT mRNA expression.

Statistical Analyses

Data from each experiment were subjected to ANOVA according to the General Linear Model (GLM). Tukey's multiple-comparison procedure (Neter et al. 1990) was used to detect significant differences among tissues and different follicular sizes. Differences in GOAT expression between various tissues and during fed and fasted conditions were considered significant when $P < 0.05$. Statistical analyses of data was completed with Minitab software (Version 14 5.1.2600, State College, PA).

RESULTS

Experiment 1 and 2

GOAT mRNA was detected by real time RT-PCR in the proventriculus, duodenum, ileum, and large intestine of 5 to 6 week old broilers (Figure 6.1). GOAT mRNA was not detected in adrenal gland, liver, or kidney tissue. Investigation into potential differential expression of GOAT within the proventriculus indicated that GOAT mRNA concentrations were

not significantly different between the proximal and distal ends of the proventriculus (Figure 6.2).

Experiment 3

GOAT mRNA was not detected in the pituitary of either fed or fasted BB hens. The mRNA expression of GOAT in proventriculus was greater in fasted versus fed broiler breeder hens (Figure 6.3). Theca tissue isolated from hierarchical and prehierarchical follicles did not have any detectable GOAT mRNA expression. GOAT mRNA was detected in granulosa tissue, but the overall mRNA expression of GOAT from granulosa tissue isolated from broiler breeder hens that were fed or fasted for 72 hours did not differ (Figures 6.4). Because there were no differences in GOAT mRNA expression between feeding state, the individual granulosa and theca mRNA expression amounts of GOAT for fed and fasted birds was combined to give a total of 6 replicate samples (3 fasted plus 3 fed) for each tissue type at each follicle size. Expression of GOAT mRNA was not detectable in granulosa cells isolated from the F_1 follicle and in only 2 (1 fasted and 1 fed sample) of the 6 replicate F_2 granulosa samples. The mRNA expression of GOAT in granulosa cells isolated from the LWF is greater than in granulosa cells isolated from the F_3 follicle (Figure 6.5).

DISCUSSION

Several novel findings resulted from the current research. This research is the first to document GOAT mRNA expression in chickens. Expression of GOAT mRNA is abundant in the proventriculus of broilers and broiler breeders but is not detected in most tissues. However, GOAT mRNA is detected in granulosa cells of preovulatory follicles and its expression decreases with follicular maturity. Fasting in broiler breeder hens is associated with an increase

in GOAT mRNA expression in the proventriculus, but not in granulosa cells isolated from any preovulatory follicle size.

In mammalian species, GOAT is produced most prominently in glandular gastric tissues (Sakata et al., 2009; Stengel et al., 2010; Kang et al., 2011; Lim et al., 2011). Differential expression of GOAT within the stomach has been reported in mice where higher concentrations of GOAT were detected by immunohistochemistry in the gastric corpus rather than the fundus (Kang et al., 2011). In the current research, no differences were detected in GOAT mRNA expression between proximal and distal portions of the proventriculus, which is the gastric stomach in avian species. The lack of a difference in GOAT mRNA expression does not preclude the possibility that differences might exist at the protein expression level. Overall, the mRNA expression profile of GOAT in broilers mirrors that reported by Gutierrez et al. (2008), who examined GOAT mRNA expression in 48 human tissues. They determined that GOAT is a message of relatively low abundance even in the two tissues with the most abundant expression: the stomach and pancreas. They detected GOAT mRNA in only a few other tissues where expression was considerably lower relative to the stomach and pancreas. Measured cycle threshold values obtained during real time PCR in the current research were 30 or less only for proventriculus, which indicates that GOAT is also a very rare transcript in broilers.

In the current research, caloric restriction of broiler breeder hens for 72 hours resulted in a significant increase in proventricular GOAT mRNA expression relative to control hens that were provisioned with their daily, restricted feed amount during the same time period. Increased concentrations of acylated plasma ghrelin have been reported in fasted mammals as well as birds (Cummings et al., 2001; Toshinai et al., 2001; Shousha et al., 2005; Kaiya et al., 2007; Freeman, 2008). The greater levels of plasma acylated ghrelin indicated that GOAT activity and/or

expression are also likely up-regulated during fasting in both mammals and birds. The current research, and research conducted in mice in which GOAT expression increased during fasting (Gahete et al., 2010), indicate that fasting increases GOAT expression.

Previously, our laboratory reported that the mRNA for the ghrelin receptor is found in the theca and granulosa cells of prehierarchical and hierarchical follicles of broiler breeder hens (Freeman, 2008). Furthermore, fasted broiler breeder hens have elevated plasma acylated ghrelin levels, and in broiler breeder hens fasted for 72 hours, the level of the mRNA for the ghrelin receptor is up regulated in theca tissue while it remains unchanged in the granulosa tissue (Freeman, 2008). The increase in GOAT mRNA levels in the proventriculus at 72 hours of fasting and the associated increase in plasma acylated ghrelin combined with the fasting induced up-regulation of theca ghrelin receptor mRNA at 72 hours suggests that ghrelin could be responsible for communicating caloric insufficiency to the developing follicles which could trigger the initiation of atresia.

The mRNA expression of GOAT in the granulosa cells of preovulatory follicles of broiler breeder hens was not affected by fasting. However, the fact that granulosa cells are one of the few tissues to even express GOAT mRNA is an interesting indication that ghrelin is an important potential regulator of follicular development. Previously we were unable to detect the mRNA for ghrelin in the broiler breeder ovary by RT-PRC (Freeman, 2008). However, in light of the current results the possibility of local production of ghrelin by the hen ovary will have to be reinvestigated with a focus on the small prehierarchical follicles as GOAT expression decreased with follicular maturation. Although local ghrelin production by granulosa cells seems the most plausible, it is possible that the granulosa cells produce acylated ghrelin using plasma born unacylated ghrelin. In all species studied, most of the ghrelin produced and found in the blood is

unacylated ghrelin. However, the granulosa cells are avascular and are situated underneath the vascular theca cells in the developing follicle. Furthermore, the cellular mechanics of acylating plasma born unacylated ghrelin would be challenging unless for example unacylated ghrelin can be translocated into the granulosa cell to be acylated and then released after acylation. The role that localized produced ghrelin would play in the developing follicle is unclear, although based on limited research in mammalian species ghrelin has been reported to affect steroid production in the testes and ovary (Muccioli et al., 2011).

In summary, the current research indicates that avian GOAT mRNA is expressed most prominently in the glandular stomach of chickens and that food deprivation up-regulates its production in the proventriculus. GOAT mRNA was also detected in developing follicles of the broiler breeder hen ovary, but its expression level did not change with fasting. The current results, combined with our previous results, suggest that ghrelin could be a key regulator of follicular development during both the fed and fasted state and provide a link between nutritional status and reproductive capability. However, further research is needed to develop this hypothesis.

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Table 6.1. Chicken GOAT oligonucleotide primer pairs for RT-PCR

Primer	Oligonucleotide Primer Sequence	Product Size (bp)
Forward 1	5’-GGGGCTGGAAGAAGCAAGGCT-3’	565
Reverse 1	5’-ACCATGATGGCCCAGCACAGGAAACC-3’	
Forward 2	5’-CAGCTACCTGCTCTTCTTCCCAGC-3’	502
Reverse 2	5’-AGGCCGTGCCACCATGCAGAGAAGGC-3’	
Forward 2	5’-CAGCTACCTGCTCTTCTTCCCAGC-3’	822
Reverse 3	5’-CAGGGACATCCTCTAGCCAGCCAG-3’	

Table 6.2. Chicken GOAT and GAPDH oligonucleotide primer pairs for real time-PCR

Product	Primer	Oligonucleotide Primer Sequence	Product Size (bp)
GOAT	Forward	5'-ACCTTTCTGGCCACGACCTT-3'	82
	Reverse	5'-GATGTACTCCTGTTCCACGTTCTG-3'	
	Probe	5'-ACACATCGCCTGGGCT-3'	
GAPDH	Forward	5'-TTGGCATTGTGGAGGGTCTT-3'	87
	Reverse	5'-GGGCCATCCACCGTCTTC-3'	
	Probe	5'-TGACCACTGTCCATGCCAT-3'	

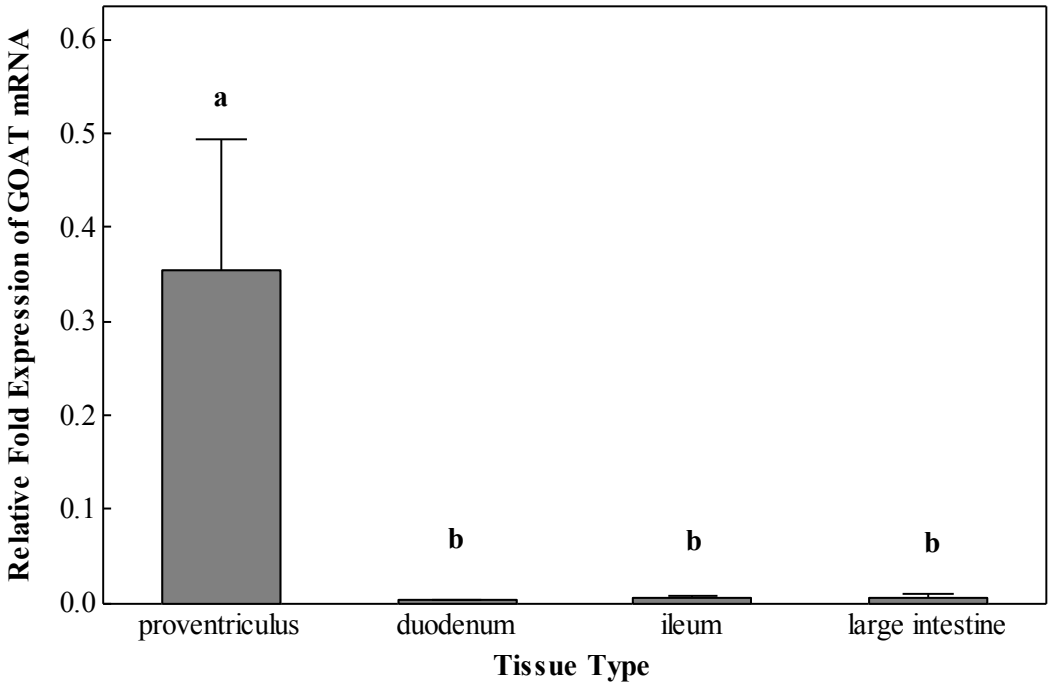

Figure 6.1. The relative fold expression of GOAT mRNA in tissues isolated from 5 week old broilers (experiment 1). The mRNA expression data were normalized with glyceraldehyde-3-phosphate dehydrogenase and expressed as the mean fold difference ($2^{-\Delta\Delta CT}$) ± SEM, n = 6. [a-b]Means with different letters differ, $P < 0.05$.

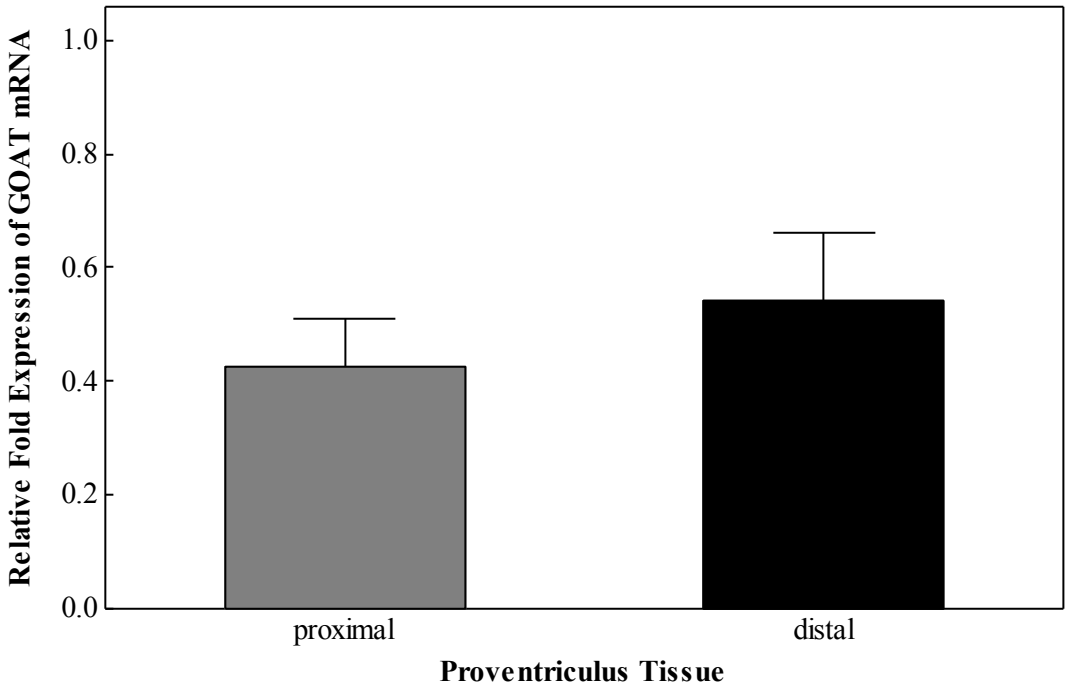

Figure 6.2. The relative fold expression of GOAT mRNA in proximal and distal proventriculus tissue isolated from 5 week old broilers (experiment 2). The mRNA expression data were normalized with glyceraldehyde-3-phosphate dehydrogenase and expressed as the mean fold difference ($2^{-\Delta\Delta CT}$) ± SEM, n = 6.

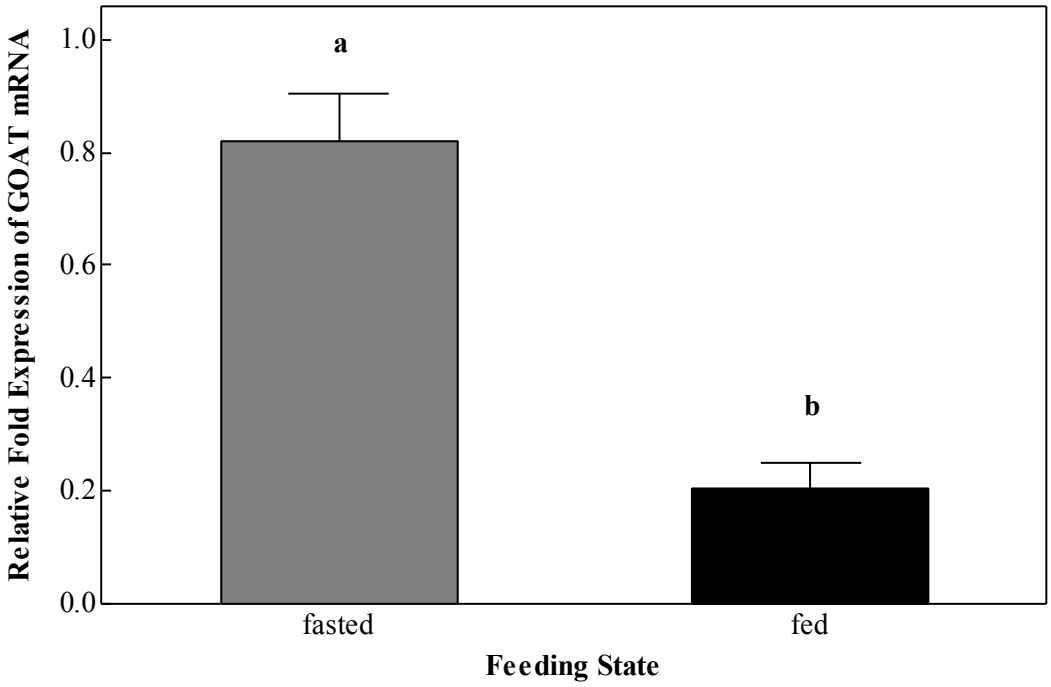

Figure 6.3. The relative fold expression of GOAT mRNA in proventriculus tissue isolated from 45 to 55 week old broiler breeder hens that were fed or fasted for 72 hours (experiment 3). The mRNA expression data were normalized with glyceraldehyde-3-phosphate dehydrogenase and expressed as the mean fold difference ($2^{-\Delta\Delta CT}$) ± SEM, n = 6. $^{a-b}$Means without a common letter differ, ($P < 0.005$).

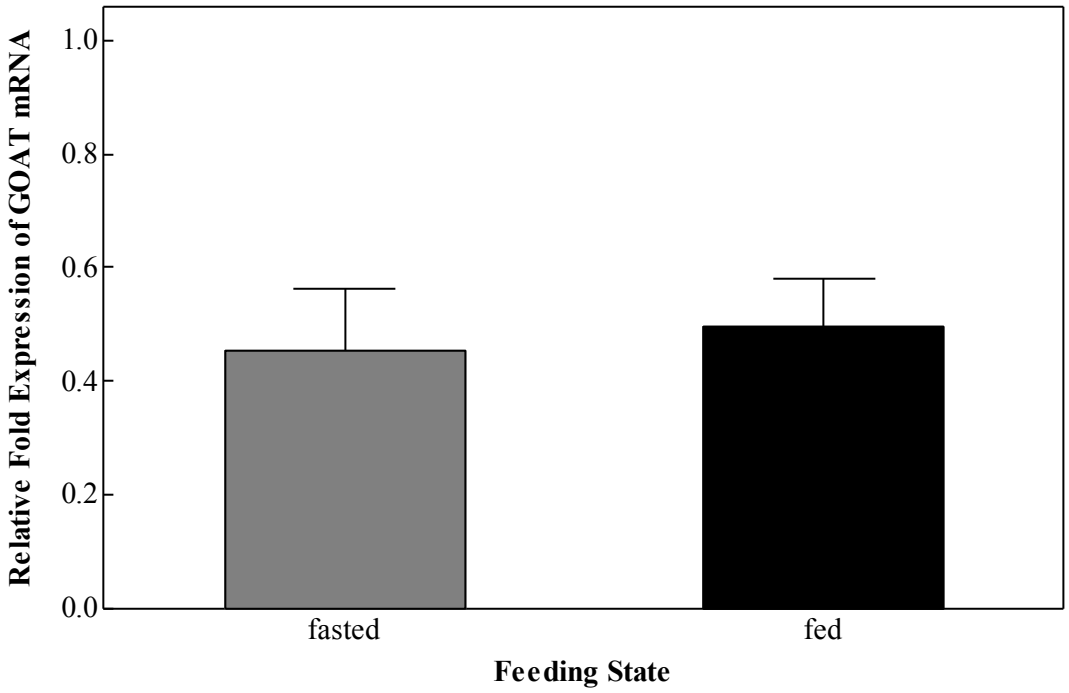

Figure 6.4. The overall relative fold expression of GOAT mRNA in granulosa tissue collected from the four largest hierarchical (F1 through F4) follicles and the small yellow and large white follicles from 45 to 55 week old broiler breeder hens fed daily or fasted for 72 hours (experiment 3). The mRNA expression data were normalized with glyceraldehyde-3-phosphate dehydrogenase and expressed as the mean fold difference ($2^{-\Delta\Delta CT}$) ± SEM, n = 18 (3 replicate birds, each with 6 follicle sizes).

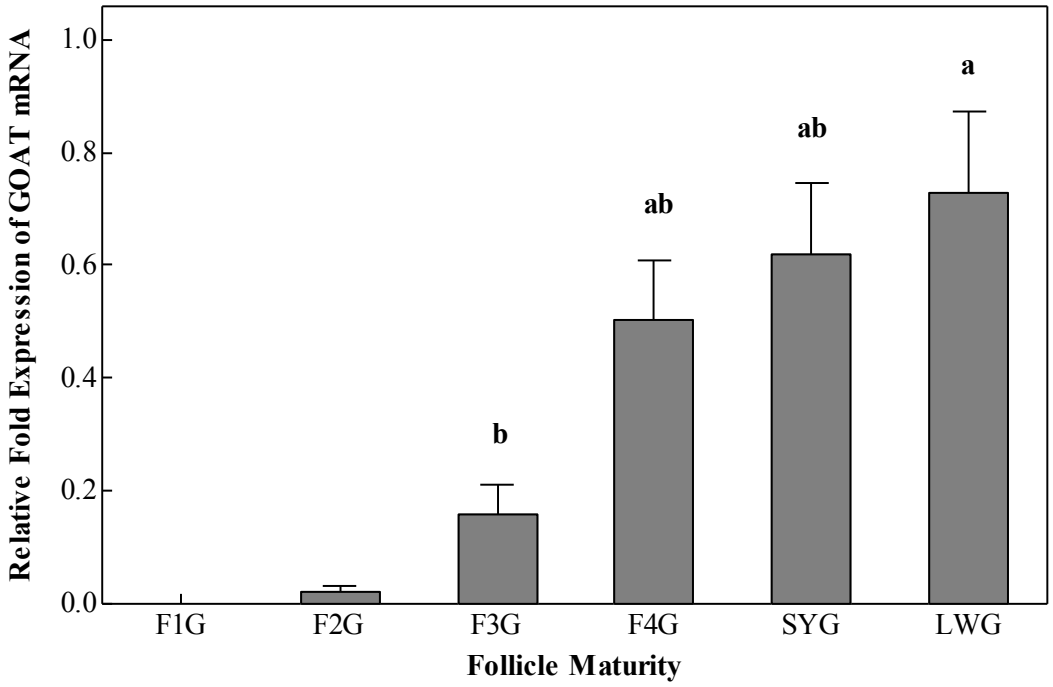

Figure 6.5. The relative fold expression of GOAT mRNA in granulosa (G) tissue collected from the four largest hierarchical follicles (F1 to F4), small yellow (SY) and large white (LW) follicles from 45 to 55 week old broiler breeder hens (experiment 3). The mRNA expression data were normalized with glyceraldehyde-3-phosphate dehydrogenase and expressed as the mean fold difference ($2^{-\Delta\Delta CT}$) ± SEM, n = 6. [a-b]Means with different letters differ, $P < 0.05$. Note that GOAT mRNA was not detected in any F1 samples and only 2 of 6 of the F2 samples. Thus, F1 and F2 relative fold GOAT mRNA expression levels were not included in the statistical comparisons.

CHAPTER 7

GENERAL OVERVIEW

Broiler Amino Acid Nutrition

In the area of poultry nutrition, more research has been devoted to amino acid requirement research than for any other nutritional aspect. However, continued research on amino acid requirements is needed due to several factors. One of the these factors is the production of synthetic amino acids, which allows poultry nutritionists to meet essential amino acid requirements through the use of these feed additives rather than meeting the requirement with natural feedstuffs. By not having to utilize natural feedstuffs to meet the limiting amino acids (methionine, lysine, and threonine) in a corn/soy diet, the total level of protein in the diet has been reduced. This is because all of the extra amino acids associated with obtaining the targeted amino acid in natural feedstuffs are eliminated when the target amino acid is simply added to the diet in a pure form.

Although the total amount of protein (amino acids) needed for broiler diets should have been reduced with the availability of synthetic amino acids, this has not been realized. This is due to the continued genetic gains made in broiler strains where with genetic selection, the number of days to reach market size continues to decrease while the final weight of broilers continues to increase and feed efficiency improves as well. Thus, the dietary density of amino acids has increased to meet the needs of today's broiler,

which grows faster, has more breast yield, and eats less than the broilers of the past. For the most part, the increase in the dietary density of amino acids needed by today's broiler has been offset by the decrease in the total amount of excess amino acids that has resulted from the use of synthetic amino acids. Meaning, that the dietary crude protein levels of broiler diets today are not that different from the past and this trend is likely to continue as new synthetic amino acids enter the market and advancements in broiler genetics continue.

The advancement in broiler genetics has resulted in new feeding regimen time periods as the duration of feeding starter (in some cases even pre-starters), grower, finisher, and withdrawal diets continues to evolve to meet the quicker growing bird and the market demands of broiler products, which favor different sized breast yields for different market purposes. When these market demands are combined with improved broiler genetics and the availability of an expanding line of synthetic amino acids, the need for continued refinement of amino acid requirements is understandable.

Another component driving amino acid requirement research is environmental concerns. Eliminating excess dietary amino acids from the diet not only saves poultry producers significant money as meeting amino acid requirements is the most expensive component of poultry diets, which diets account for about 70% of broiler production costs, but it also reduces pollution. A reduction of excess dietary amino acids will decrease unwanted nitrogen in poultry waste. Higher nitrogen levels in poultry litter can lead to more ammonia production, which can decrease bird health and cause ammonia emission from the broiler production unit. Furthermore, the nitrogen from litter, while a good fertilizer, has the potential when applied to crop lands in some situations to leach

into watersheds and cause eutrophication and serious environmental pollution problems. The trend by poultry nutritionists to formulate diets based on digestible amino acid requirements rather than total amino acid requirements ultimately better matches the bird's exact amino acid needs with what has to be delivered from the diet. Thus, again reducing the amount of nitrogen found in litter.

The current research on dLys and dThr requirements fit perfectly into the demands of amino acid requirements. The dLys requirement calculated in two genetic crosses of broilers from 35 to 49 days of age was needed as it becomes only the second research report overall and the first with these strains to define the dLys requirement during this critical period. The number of birds raised to 49 days has increased to meet the demands of the breast products produced by these sized birds. Additionally, there had been a trend by poultry nutritionists to increase dietary dThr levels based on two research reports, which indicated that the increased levels of dLys used to maximize breast yield increased the dThr requirement. The current research indicates that increasing the dThr levels in starter and grower diets is not warranted and thus, poultry nutritionists do not need to add extra threonine during this period.

Avian Ghrelin-GOAT System

One decade has passed since avian ghrelin was first isolated and cloned by Kaiya and colleagues (2002). Since then, ghrelin in chickens has been identified as a 26 amino acid-long hormone that does not share much sequence homology with mammals except for the first seven amino acids of the N-terminal sequence. The seven amino acids are highly conserved across vertebrate species and are considered the active core of the hormone. Similarly to mammals, avian ghrelin is predominantly produced by the

glandular gastric tissue, the proventriculus, and is found in two forms: des-acylated and acylated ghrelin. The latter form is covalently modified with a fatty acid moiety, which is catalyzed by the ER associated, membrane bound enzyme, GOAT. Like its substrate, ghrelin, GOAT is also highly conserved across vertebrates. In mammals, the ghrelin-GOAT system has been studied extensively and has been implicated in mediating the signal for nutrient intake between the peripheral tissues of the body and the CNS. Research conducted in murine and avian models suggests that acylated ghrelin effects feeding behavior. Furthermore, caloric restriction in rodents leads to higher mRNA expression of both ghrelin and GOAT in the stomach commensurate higher acylated and total plasma ghrelin in blood circulation. In birds, ghrelin follows a similar pattern, but prior to this research GOAT had not been characterized in birds.

The current research indicates that GOAT mRNA is primarily expressed in the proventriculus and that its expression is increased with fasting. This correlates well with the previous research from our laboratory, which indicated that acylated ghrelin levels increase with fasting. In the current research, GOAT mRNA was also detected in the granulosa cells of preovulatory follicles from broiler breeder hens. This adds further indication that ghrelin may play an important role in follicular maturation in broiler breeder hens as our laboratory had already discovered that the ghrelin receptor was expressed by the theca and granulosa cells of the preovulatory follicles and that fasting up-regulated the ghrelin receptor expression in the theca cells.

When considering the potential role of ghrelin and GOAT in regulating metabolism and feed intake in birds, the implications for the broiler industry are tremendous. Feed intake is directly correlated to body weight gain in broilers and even

the slightest improvements in carcass yield can translate into substantial economic gains for the industry. Moreover, weight gain efficiency can be further amplified by supplying broilers with balanced diets that meet the optimal amino acid requirement patterns for maximum muscle accretion.

On the other hand, for broiler breeders, further understanding of the ghrelin-GOAT axis may lead to better feed restriction strategies that minimize fasting periods between meals. Parent and grandparent broiler breeders are perpetually selected for heavier body-types and higher appetites, which are desirable traits for their offspring but detrimental for reproductive efficiency. Because of their propensity to eat continuously and become large quickly, broiler breeders have to be severely feed restricted so that they have an appropriate body weight for reproduction at 20 weeks of age. While feed restricted birds have significantly better egg production than unrestricted birds, the amount of eggs they produce per bird could increase by at least another 100 eggs based on having ovarian follicular contents similar to laying hens.

Typically in the United States, when broiler breeders are feed restricted they are fed once every other day during rearing and once a day during egg production. They consume their feed rapidly and thus, have substantial fasting periods. During these fasts, ghrelin production increases and the current research and our previous research suggests that the biological mechanisms are in place so that ghrelin might signal caloric insufficiency to the ovary and stimulate the atresia that plagues ovarian development and decreases egg production in broiler breeder hens. If this is proven to be true, management practices will need to change to eliminate the fasting periods currently associated with broiler breeder feed restriction programs.

These management changes could lead to improvements in broiler breeder reproductive efficiency. Even a marginal improvement could significantly impact the poultry industry by reducing the total number of hens needed in a breeder flock. Growing fewer but more efficient broiler breeders, lowers feed cost, housing expenditures, and reduces the industry's environmental impact.

APPENDIX

HUMAN AND RAT ABC TRANSPORTER EFFLUX OF BISPHENOL A AND BISPHENOL A GLUCURONIDE: INTERSPECIES COMPARISON AND IMPLICATIONS FOR PHARMACOKINETIC ASSESSMENT[1]

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ABSTRACT

Significant interspecies differences exist between human and rodent with respect to the absorption, distribution, and excretion of bisphenol A (BPA) and its primary metabolite, BPA-glucuronide (BPA-G). ATP-Binding Cassette (ABC) transporter enzymes play important roles in these physiological processes, and their enzyme localization (apical versus basolateral) in the plasma membrane allows for different cellular efflux pathways. In this study, we utilized an ATPase assay to evaluate BPA and BPA-G as potential substrates for the human and rat ABC transporters: P-glycoprotein (P-gp, MDR1), multidrug resistant-associated proteins (MRPs) and breast cancer-resistant protein (BCRP). Based on high ATPase activity, BPA is likely a substrate for rat mdr1b, but not for human MDR1 or rat mdr1a. Results indicate BPA is a potential substrate for rat mrp2 and human MRP2, BCRP, and MRP3. The metabolite BPA-G demonstrated the highest apparent substrate binding affinity for rat mrp2 and human MRP3, but appeared to be a non-substrate or potential inhibitor for human MRP2, MDR1, and BCRP, and rat mdr1a, mdr1b, and bcrp. Analysis of ABC transporter amino acid sequences revealed key differences in putative binding site composition that may explain substrate specificity. Collectively, these results suggest in both rat and human, apical transporters efflux BPA into the bile and/or intestinal lumen. BPA-G would follow a similar pathway in rat; however, in human, due to the basolateral location of MRP3, BPA-G would likely enter systemic and portal blood supplies. These differences between human and rodent ABC transporters may have significant implications for interspecies extrapolation used in risk assessment.

INTRODUCTION

Bisphenol A [4,4'-(propane-2,2-diyl)diphenol, BPA] is a high-production volume chemical used in the manufacture of polycarbonate plastics and epoxy resins (Teeguarden et al., 2005; Volkel et al. 2005). Widespread inclusion of BPA in consumer products, including the protective lining of metal food cans, polyvinyl chloride pipes, and shatter-proof plastic bottles has greatly enhanced the potential for human exposure (Pritchett et al., 2002; Doerge, 2010). While BPA is classified as a suspected endocrine-disrupting chemical which displays aberrant reproductive and developmental effects in laboratory animals, safety concerns regarding low-dose human exposure remain controversial (Volkel et al., 2002).

Xenobiotic pharmacokinetics involve dynamic processes including uptake, distribution, metabolism, and excretion of the parent chemical and its metabolite(s) (Pang, 2009). In humans and rodents, BPA undergoes extensive phase II metabolism, via uridine diphosphate-glucuronosyl transferase (UGT) conjugation, to form the metabolite, BPA-glucuronide (BPA-G) (Yokota et al., 1999; Pritchett et al., 2002; Kuester and Sipes, 2007). In general, BPA conjugation first occurs pre-systemically in the gastrointestinal tract, followed by conjugation in the liver during first-pass metabolism (Mazur et al., 2010; Fisher, 2012).

Interspecies metabolic differences exist between primates and rodents regarding the excretion of BPA and BPA-G. In human studies, administered BPA is predominantly recovered as the BPA-G metabolite in urine, while in rats, the biliary excretion of BPA predominates, with a small percentage of BPA-G excreted in the urine (Volkel et al., 2002; Inoue et al., 2003; Takeuchi et al., 2004; Teeguarden et al., 2005). The physicochemical properties of xenobiotics and their metabolites help dictate their biological fate and kinetics, including entry (influx) and exit (efflux) across cellular membranes (Oswald et al., 2007). Because BPA is uncharged,

relatively small, and moderately hydrophobic, its passive diffusion across cellular membranes is favored (Mørck et al., 2010). In contrast, BPA-G is a hydrophilic anion that often requires mediated transport to cross cellular membranes (Zamek-Gliszczynski, 2011).

It is well established that active transport can be a major pharmacokinetic determinant in the disposition and metabolism of xenobiotics. (Kim, 2006). The ATP-Binding Cassette (ABC) superfamily is composed of ATP-hydrolyzing enzymes that actively transport a broad range of substrates (Glavinas et al., 2004). Cytoplasmic ATP-binding domains harness energy from ATP hydrolysis for substrate transport across membranes, while membrane-spanning domains, composed of multiple transmembrane (TM) α-helices, form the substrate binding pocket (Aller et al., 2009). Specific amino acids within or near transporter binding pockets have been shown to play important roles in transporter-specific substrate specificity based on hydrophobic, aromatic and noncovalent interactions (Karlsson et al., 2010).

ABC efflux transporters are expressed at high levels in organs of excretion such as the intestine, liver, kidney and placenta (Oswald et al., 2007; Prouillac, 2010). It is widely believed that their predominant role is to prevent the cellular accumulation of potentially toxic substances (Oswald et al., 2007). However, localization of these transporters on the apical or basolateral side of plasma membranes is an important determinant of xenobiotic transport. In the liver, efflux transporters expressed on the apical side of hepatocyte membranes will efflux xenobiotics out of the cell and into the bile, while those expressed on the basolateral side will efflux xenobiotics to the blood. In the placenta, membrane localization can be a critical determinant of fetal exposure through the efflux of xenobiotics into the fetal circulation. Additionally, interspecies differences in transporter-based efflux in the small intestine and liver may alter the kinetics of first-pass metabolism or systemic clearance of xenobiotics.

BPA metabolism is often studied in vitro using subcellular microsomal fractions or isolated cryopreserved primary hepatocytes (Elsby et al., 2001; Pritchett et al., 2002; Kuester and Sipes, 2007; Mazur et al., 2010). While such techniques are useful for high-throughput pharmacokinetic analyses, the cellular machinery that allows efflux transport in vivo is rapidly disrupted when cells or subcellular fractions are isolated from the intact organ (Csala et al., 2004; Bow et al., 2008; Li et al., 2009). ATPase assays utilize membranes with functioning transporters and allow for the rapid screening and identification of potential substrates and inhibitors.

Given that ABC transporters play a key role in determining the exposure of various organs and tissues to a variety of environmental toxins, drugs and other xenobiotics, a better understanding of their substrate specificity, cellular localization and interspecies differences is necessary. This study investigates the interaction of BPA and its primary metabolite, BPA-G, in vitro with specific members of three major subfamilies of human and rat ABC efflux transporters: P-glycoprotein (MDR1), various multidrug resistance associated proteins (MRPs), and breast cancer resistant protein (BCRP). To mechanistically evaluate species- and isoform-related differences in substrate specificity, ABC transporter amino acid sequences were analyzed at known substrate binding and recognition sites to identify differences that may impact BPA and BPA-G transport.

MATERIALS AND METHODS

Reagents

BPA (>99% purity) and acetonitrile (>99% purity) were purchased from Sigma-Aldrich (St. Louis, MO); BPA-G (>98 % purity) was a gift from the National Institute of Environmental Health Sciences (Research Triangle Park, NC). All chemical reagents, selective ATPase activity inhibitors (orthovanadate) and transporter substrates (verapamil, sulfasalazine, probenecid and benzbromarone) were included in the ATPase Assay Kits purchased from BD Biosciences Discovery (Woburn, MA).

ATPase Activity Assays

Baculovirus-infected insect cells expressing human MDR1, BCRP, MRP2, MRP3 and rat mdr1a, mdr1b and bcrp and mrp2 transporter isoforms were purchased from BD Biosciences Discovery Labware (Woburn, MA). Membrane preparations and ATPase assay techniques were used according to Sarkadi et al. with slight modifications (Sarkadi et al., 1992; Drueckes et al., 1995). Insect cell membranes were stored at -80˚C until use. ATPase assay buffer was comprised of 50 mM Tris-Mes buffer (pH 6.8), 50 mM KCl, 2 mM DTT, 2 mM EGTA, and 5 mM sodium azide and stored at -20˚C until use. BPA and BPA-G stock solutions (60 mM) were prepared in acetonitrile and subsequent dilutions (1.95- 62.5 µM) were prepared using ATPase assay buffer containing 2.5% acetonitrile; final acetonitrile concentration in ATPase assay reactions wells was 0.83%. To perform the ATPase assay, cell membranes were first thawed in a 37˚C shaking water bath and diluted to 1 mg/mL using assay buffer. Cell membranes were split into two samples with 400 µM (final concentration) of the inhibitor, orthovanadate, added to one sample while an equal volume of ultra-pure water was added to the other. Transporter activity was measured as the orthovanadate-sensitive portion of the total ATPase activity. Cell membrane samples (20

μL) were loaded in triplicate onto 96-well flat bottom plates (Microtest™ Franklin, NJ) with and without orthovanadate. Serial dilutions of test chemicals and positive control substrates (20 μL) were added to the wells sequentially and incubated for 5 min at 37˚C while shaking (BioTek Synergy HT plate reader; Winooski, VT). The ATPase activity reaction was initiated by adding 20 μL (4 mM final concentration) MgATP to each well, followed by 10-60 min of shaking incubation at 37˚C (based on each transporter protocol). Reactions were stopped by adding 10% SDS (30 μL) to each well. Liberation of inorganic phosphate was determined by adding 200 μL of detection reagent (2.5 mL of 70 mM ammonium molybdate pH 5.0 and 2.5 mL of 30 mM zinc acetate pH 5.0, mixed with 20 mL of 10% ascorbic acid, pH 5.0) to each cell, followed by a 20 min shaking incubation at 37˚C. The inorganic phosphate complex was detected by its absorbance at 800 nm.

ATPase Activity Data Analysis

Baseline optical density was determined by subtracting the mean absorbance from triplicate wells with no substrate, and inhibited with orthovanadate from wells containing no substrate or inhibitor. Triplicate control values of samples containing the transporter substrate, and inhibited with orthovanadate, were subtracted from control samples containing substrate and no inhibitor. The difference in ATPase activity in the presence or absence of the inhibitor (orthovanadate) represents the transporter-mediated activity. Baseline optical density was subtracted from the net value, determined with the positive control substrate, to give maximum optical density. Maximal ATPase activity was determined for each transporter using strong, specific activators as positive controls. ATPase activity for positive controls was set to 100% and each test compound was measured as a percent ATPase stimulation of the positive controls. According to protocol, verapamil, sulfasalazine, probenecid and benzbromarone (10-1000 μM)

served as positive controls for rat and human MDR1, BCRP, MRP2, and MRP3, respectively. All test samples with BPA and BPA-G at varying concentrations were run in triplicate, with and without orthovanadate, and the net difference in absorbance was divided by the maximum optical density to give % stimulation of ATPase activity. Net positive stimulatory activity was indicative of an apparent substrate, while values within standard error of the baseline threshold were considered non-substrates. ATPase activities significantly below baseline threshold were classified as potential inhibitors.

Protein sequence analyses

Rodent and human MDR1(P-gp) and MRP protein amino acid sequences were obtained from Genbank (NCBI). Sequence homology and alignment were performed using the T-Coffee program (Notredame et al., 2000). Protein sequences were analyzed at distinct amino acids that have been reported to impact transporter substrate selectivity. A number of specific amino acid differences were identified among the transporters studied that may be modulating transporter affinity for BPA and BPA-G.

RESULTS

Characterization of ATPase Activities

BPA and its metabolite BPA-G were assessed for efflux transport specificities among rat mdr1a, mdr1b, bcrp, mrp2, and human MDR1, BCRP, MRP2, and MRP3 transporters. It is important to note that even in the absence of a substrate, baseline ATPase activity exists in transporter membranes. Thus, it is possible to achieve negative ATPase activity percent values below baseline in the presence of a slowly transported substrate or potential inhibitor.

With respect to the MDR1 subfamily, our results demonstrated markedly different ATPase activation profiles for BPA among human MDR1, rat mdr1a and rat mdr1b (Figure. 1). Human MDR1 displayed little BPA-stimulated ATPase activity (0-62.5 μM) with the highest stimulation (approximately 20% of positive control) occurring at 30 μM. BPA (0-62.5 μM) appeared to have an overall inhibitory effect on the rat mdr1a transporter as it displayed little to no ATPase activity. However, BPA with rat mdr1b demonstrated a significant increase in ATPase activity to above 60% of positive control. BPA-stimulated rat mdr1b ATPase activity followed the bell-shaped curve commonly observed among MDR1 substrates (Buxbaum, 1999), with maximum activity at approximately 15 μM BPA.

Significant differences in ATPase activity were also observed among the MRP subfamily in both rats and humans towards the metabolite BPA-G. Human MRP3 displayed high ATPase activity towards BPA-G to above 80% of positive control, with a continuous increase in stimulation that correlated with increasing BPA-G concentration (0-62.5 μM; Figure. 2). Rat mrp2 displayed low to moderate stimulation (approximately 30% of positive control) in response to BPA-G at concentrations greater than 30 μM, while human MRP2 showed little to no stimulation, with a slight inhibitory profile, in response to BPA-G. A similar trend occurred in these MRP transporters with respect to BPA. While human MRP3 and rat mrp2 showed moderate ATPase stimulation (upwards of 30% of positive control) with the parent compound BPA, human MRP2 showed little BPA-induced ATPase stimulation (<10% of positive control) (Representative activity profiles shown at 30 μM, Figure. 3A).

The BCRP subfamily of ABC transporters displayed slight differences in response to BPA, with human BCRP showing higher ATPase activity (approximately 20% of positive control at 30 μm BPA) than rat bcrp, which displayed little to no stimulation (<10% of positive

control at 30 μm BPA; Figure. 3A). In response to the metabolite BPA-G, both rat and human BCRP demonstrated little stimulation (<5% of positive control; Figure. 3B), indicating BPA-G is likely not a substrate for the rat and human BCRP isoforms. These results are similar to that observed for the rat and human MDR1 isoforms in which BPA-G also did not appear to be a substrate, rather appearing to inhibit the ATPase activity of human MDR 1 and rat mdr1a and mdr1b (Figure. 3B).

Transporter Amino Acid Sequence Analyses

In this study, initial protein amino acid sequence comparisons revealed that the full length human MDR1 sequence is 87% and 80% identical to the rat mdr1a and rat mdr1b isoforms, respectively, while the full length rat mdr1a and rat mdr1b sequences were found to be 84% identical. Human MRP2 was found to have 78% and 46% amino acid sequence identity with the rat mrp2 and human MRP3 isoforms, respectively, while rat mrp2 and human MRP3 were found to be 45% sequence identical. Among these isoforms, protein amino acid sequence analyses confirmed the presence of known highly conserved amino acids required for general ABC transporter function including those present in the ABC signature and Walkers A and B motifs.

Protein sequences were further analyzed to identify non-conserved amino acids in putative ABC transporter substrate binding regions that may have key roles in substrate specificity. These amino acid differences among the rat and human MDR1 isoforms were found exclusively in the transmembrane (TM) helical binding pocket regions and included Gly64 (amino acid numbering based on the human MDR1 protein sequence), Ser196, Ile306, Val338, Ile340, Ala342, Leu762, Ser943, Leu975, Val981, and Gln990 (Figure.4A). Among the rat and human MRP isoforms, differences at amino acids that may be a factor in substrate specificity were also identified and included Cys208 (amino acid numbering based on the human MRP1

protein sequence), Glu210, Trp261, Lys267, Lys319, Lys347, Pro448, Trp553 and Phe594 (Figure. 4B).

DISCUSSION

It is well established that ABC transporters play a fundamental role in the absorption, distribution, metabolism and excretion of endogenous and exogenous chemicals, and transporter membrane localization can directly influence these processes (Glavinas et al., 2004). The apical localization of efflux transporters in the liver and intestine facilitates biliary excretion and substrate transport back into the intestinal lumen, respectively, while basolateral localization in both liver and intestine facilitates efflux of the substrate into blood circulation (Figure.5) (Leslie, 2005). Insight into the physiological role of ABC transporters is critical for the pharmacokinetic assessment of BPA. Moreover, delineating inter- and intraspecies differences of BPA and BPA-G efflux is necessary to reduce uncertainties associated with allometric scaling of rodent kinetic parameters for human applications.

ABC efflux transporters use ATP as an energy source to transport substrates across cell membranes, and it is generally accepted that substrate-stimulated ATPase activity correlates well with substrate transport (Ambudkar et al., 1997; Glavinas et al., 2004). Important interspecies differences in the efflux transporter specificities for BPA and BPA-G between rat and human ABC transporters were demonstrated in this study. ATP assays conducted with human P-gp (MDR1) and rat P-gp (mdr1a and mdr1b), which are apically localized, showed significant differences in BPA-stimulated ATPase activity. Among these P-gp isoforms, rat mdr1b demonstrated the highest apparent affinity for BPA as a substrate compared to human MDR1 or

its rat ortholog, mdr1a. These results suggest a physiological preference for BPA efflux into the intestinal lumen and/or hepatobilliary excretion in rat.

Conflicting evidence exists regarding the efflux of BPA by P-gp. Using Caco-2 cells, BPA was previously reported to be a P-gp substrate (Jin and Audus, 2005), while human P-gp ATPase assays with limited BPA concentrations showed no stimulation of activity (Yoshikawa et al., 2002). To our knowledge, this is the first report assessing the potential role of the different rat P-gp isoforms (mdr1a and mdr1b) to efflux BPA. To date, the influence of efflux transporters has yet to be evaluated within established PBPK models which use BPA rat liver metabolism data scaled for human applications (Teeguarden et al., 2005). Information about the transporter-mediated efflux kinetics of BPA can be used to parameterize the mechanistic components of such PBPK models more accurately.

While P-gp (MDR1) transports a wide variety of structurally unrelated compounds, substrate specificity has been reported (Tang-Wai et al., 1995). Although highly conserved P-gp amino acids are critical for general ATP hydrolysis and transporter function, differences in non-conserved amino acids positioned within or near the binding pocket appear to play a critical role in substrate specificity via the introduction of hydrogen bonds, charged groups, differences in size and/or hydrophobicity. These changes can result in large changes in substrate binding affinity because unfavorable contacts may reduce optimal interaction. Through mutational and structure-function studies, a number of amino acids involved in P-gp substrate specificity have been identified (Loo and Clarke, 1994; Hafkemeyer et al., 1998; Aller et al., 2009). Our results revealed that rat mdr1b differs from human MDR1 and rat mdr1a at a number of these amino acids (Figure. 4A). In TM8 and TM12 of human MDR1 and rat mdr1a, two leucine residues appear to play key roles in substrate-protein interaction, while rat mdr1b has methionine residues

at these positions (M759 and M972) (Hafkemeyer et al., 1998; Aller et al., 2009). Methionine sulfur atoms can form unique interactions with non-protein constituents and are highly susceptible to oxidation, which can lead to changes in substrate binding pocket conformation. In TM11 of human MDR1 and rat mdr1a, a serine residue is believed to directly modulate substrate binding, and mutation to an alanine (the residue present in rat mdr1b-A940) leads to alterations in substrate specificity. Collectively, we hypothesize that the substrate-binding pocket of rat mdr1b differs from human MDR1 and rat mdr1a both in primary sequence and higher order structure, which may be the molecular basis underlying the specificity of rat mdr1b for BPA .

Upon entering a hepatocyte or enterocyte, BPA may be either effluxed out of the cell or metabolized to BPA-G (Figure. 5). Comparing our results for BPA-induced stimulation of ATPase activity with efflux proteins from the MRP and BCRP family, the rat mrp2 isoform demonstrated higher activity than human MRP2, whereas BCRP was the sole efflux transporter for which human ATPase activity was greater than rat. While identifying positive stimulation of efflux transporters is critical, knowledge of possible inhibition or slow transport interactions that lower baseline activity (below 0%) is important to understanding possible chemical-chemical interactions. Out of the eight different rat and human efflux transporter assays evaluated in this study, six assays conducted with the BPA-G metabolite demonstrated ATPase activity below the baseline. With the anionic BPA-G, neither rat nor human MDR1 or BCRP isoform(s) demonstrated detectable efflux transport. The observed decrease below baseline activity may indicate potential inhibitory effects and requires further investigation (Giacomini et al., 2010). Importantly, inhibiting efflux transport processes may also adversely affect target dose concentrations of therapeutic agents or clearance capacity for other xenobiotic exposures.

The MRP subfamily has a general affinity to efflux hydrophilic organic anions including glucuronate and glutathione conjugates (Glavinas et al., 2004). Our results indicate significant inter- and intraspecies differences towards BPA-G were observed between MRP2 and MRP3. Human MRP3 demonstrated the highest apparent substrate affinity for BPA-G (rat mrp3 was unavailable for purchase), suggesting potential basolateral transport preference of this metabolite into the blood supply. Among MRP2 isoforms, rat isoforms demonstrated higher BPA-G-induced ATPase stimulation than human isoforms. These results indicate preference for hepatobiliary or intestinal lumen excretion within rat versus human. The implication of these findings is highly significant in light of a recent report indicating expression of MRP2 is approximately 10-fold higher in rat liver than either monkey or human livers (Li, 2009). In liver and intestine, the apical localization of MRP2 would facilitate efflux back into the intestinal lumen or excretion into bile within the liver, while the basolateral localization of human MPR3 would provide a pathway entrance into systemic blood flow.

Currently, the MRP family of transporters is not well characterized and the majority of MRP substrate specificity studies involve human MRP1 amino acids (Koike et al., 2002; Campbell et al., 2004; Haimeur et al., 2004; Koike et al., 2004; Leslie, 2005). In this study, we identified MRP3 isoform-specific differences at specific amino acids that may be a factor in MRP3 substrate specificity for BPA-G (Figure 4B). For example, Human MRP3 was found to differ from both human MRP2 and rat mrp2 with the presence of two tryptophan amino acids at positions 260 and 539. Due to their bulky aromatic side chains, these amino acids are believed to contribute significantly to MRP substrate specificity by altering the structure of the substrate binding pocket (Ren et al., 2001). These and other differences in the human MRP3 amino acid

sequence support our findings that human MRP3 is distinct in its apparent preference for BPA-G.

Remarkable physiological differences exist between primates and rodents in the pharmacokinetic behavior of BPA. In vivo studies indicate that the bioavailability of orally administered BPA is low in rats, monkeys and humans (Volkel et al., 2002; Doerge et al., 2011; Doerge et al., 2011). In humans and non-human primates (monkeys), ingested BPA is rapidly metabolized in the gastrointestinal tract and liver, and over 95% of the BPA is excreted in urine as BPA-G; only a few percent (2-3%) of an orally administered dose of BPA was accounted for in feces of monkeys (Kurebayashi et al., 2002). In contrast, rodent studies show most (85%) BPA is eliminated in feces as BPA, while the remainder (15%) is excreted in urine as BPA-G (Pottenger et al., 2000).

Understanding the role and impact of transporter proteins on the disposition of xenobiotics such as BPA and BPA-G will help provide a mechanistic framework to describe the influx and efflux of materials in target organs in PBPK models. Characterizing active transport in PBPK models becomes important when concentration gradients are observed between blood and an organ that cannot be described by thermodynamic properties (i.e., tissue:blood partition coefficent) or other interactions, such as nonspecific binding. In the case of BPA-G, transporters may be the primary reason for differences in excretion between rodents and primates. Furthermore, potential BPA-G inhibition of other transport processes could alter the efflux pharmacokinetics of the parent BPA in both the liver and intestine, thus changing its intracellular concentration and possibly driving BPA efflux via alternate uninhibited transport pathways (Figure. 5).

In summary, our results indicate BPA generally stimulates the ATPase activity of the rat apical transporters mdr1b, mrp2 and bcrp, while BPA-G stimulates mrp2, suggesting possible transport preferences of both BPA and BPA-G into the intestinal lumen and hepatobiliary excretion in rats. In humans, BPA induces increased ATPase stimulation for the efflux transporters MDR1, MRP2, and BCRP located apically and MRP3 located basolaterally (Figure. 5). MRP3 showed the highest ATPase activity in response to BPA-G with no stimulation of the apical transporters. This suggests possible transport preferences of BPA-G into the blood supply of the liver or portal blood supply of the small intestine in humans. Our human MRP3 data support assumptions used by Fisher et al. to describe the pharmacokinetics of orally administered BPA in monkeys and humans using a PBPK model (Fisher et al., 2011). For the BPA PBPK model, extensive metabolism of BPA to BPA-G was predicted in the small intestine (enterocytes), and the systemic uptake of BPA-G from the intestine was described using a large first-order rate constant, consistent with active transport of BPA-G from the enterocytes into the blood supply. Further interspecies studies are needed to address MRP differences across rodents themselves, since mrp3-deficient mice dosed with BPA demonstrate lower BPA-G levels compared to wild-type (Hirouchi et al., 2009).

Additional in vitro transport studies are needed to assess BPA systemic clearance adequately. Following the identification of key transporter specificities for BPA and BPA-G through ATPase assay measurements, other techniques including membrane vesicles and cell-based assay systems can be carried out to delineate the quantitative aspects of the bi-directional transport kinetics of these compounds at environmentally relevant concentrations (Giacomini et al., 2010). Clarifying the histological localization for many transporter proteins is necessary to understand physiological function and may prove critical for assessing BPA fetal-placental

transfer (Prouillac, 2010). Furthermore, future PBPK exposure models assimilated with high-throughput in vitro clearance data, derived using subcellular liver microsomes or hepatocyte suspensions which have limited transport activities, must evaluate whether transport kinetics are the rate-determining step in hepatic elimination (Csala et al., 2004; Hirouchi et al., 2009; Li et al., 2009; Wetmore et al., 2012).

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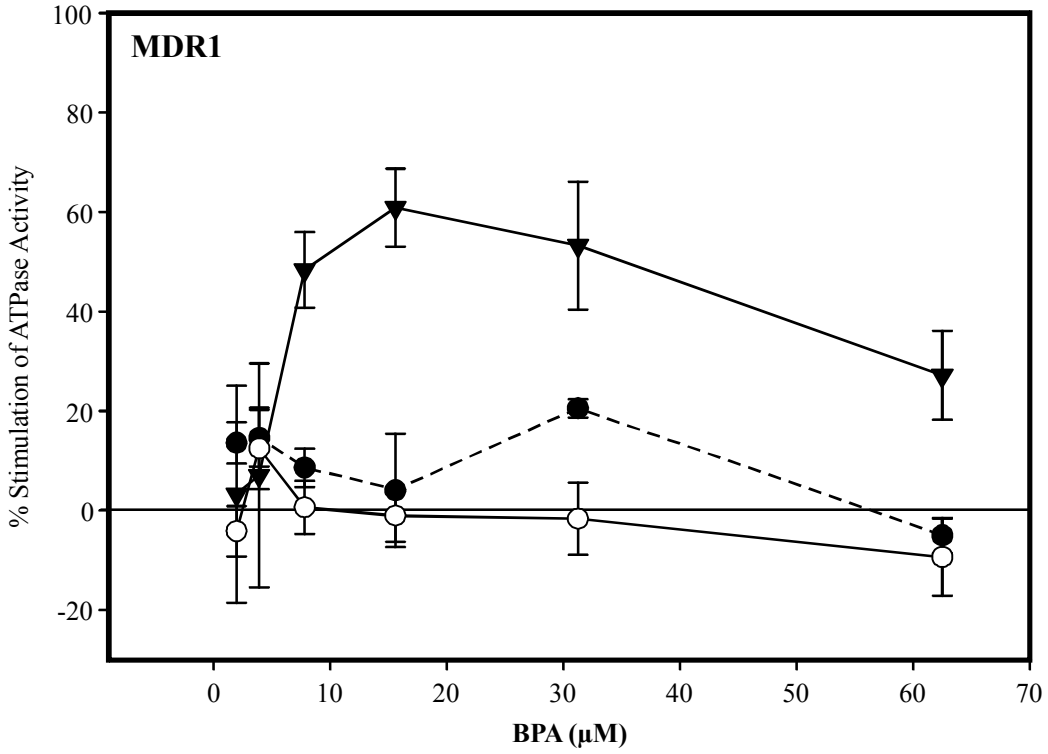


Figure 1

Figure 1. Bisphenol A (BPA) stimulation of P-gp (MDR1) ATPase activity with human MDR1 (●), rat mdr1a (○), and rat mdr1b (▼) isoforms.

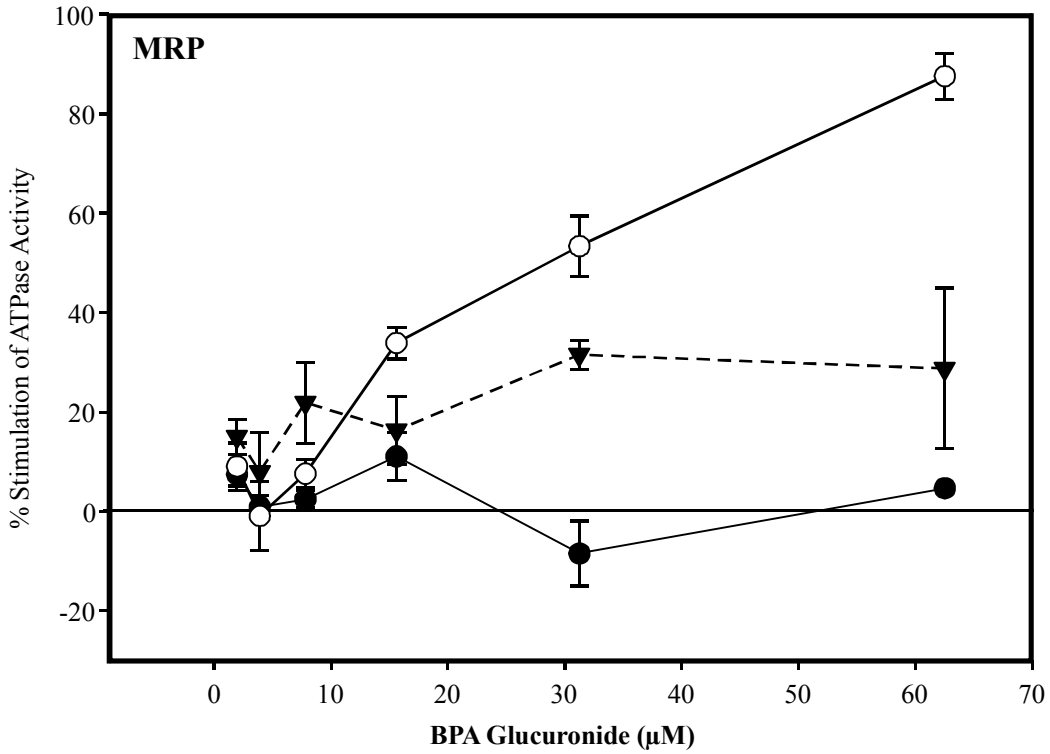


Figure 2

Figure 2. Bisphenol A glucuronide (BPA-G) stimulation of MRP ATPase activity with human MRP2 (●), human MRP3 (○), and rat mrp2 (▼) isoforms.

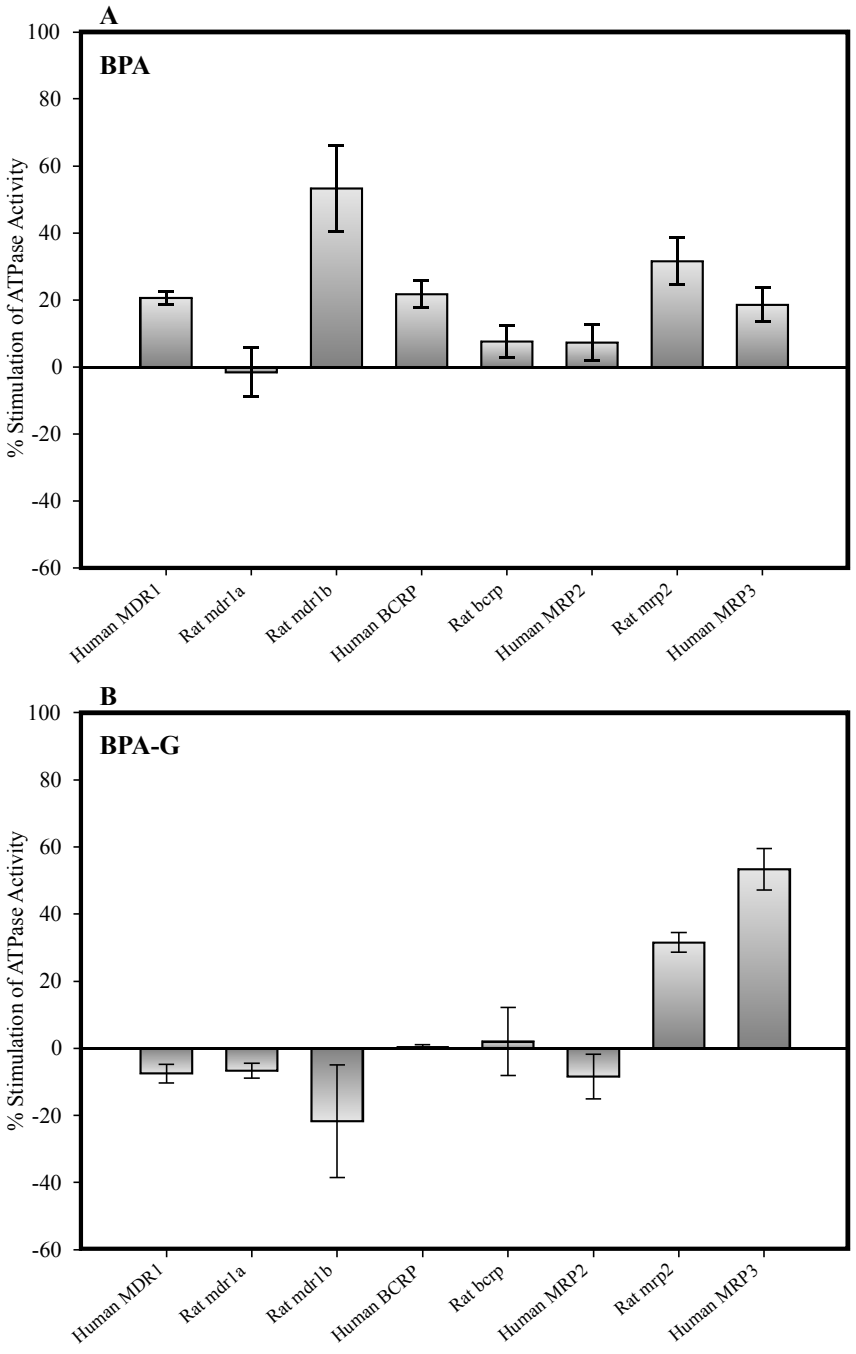
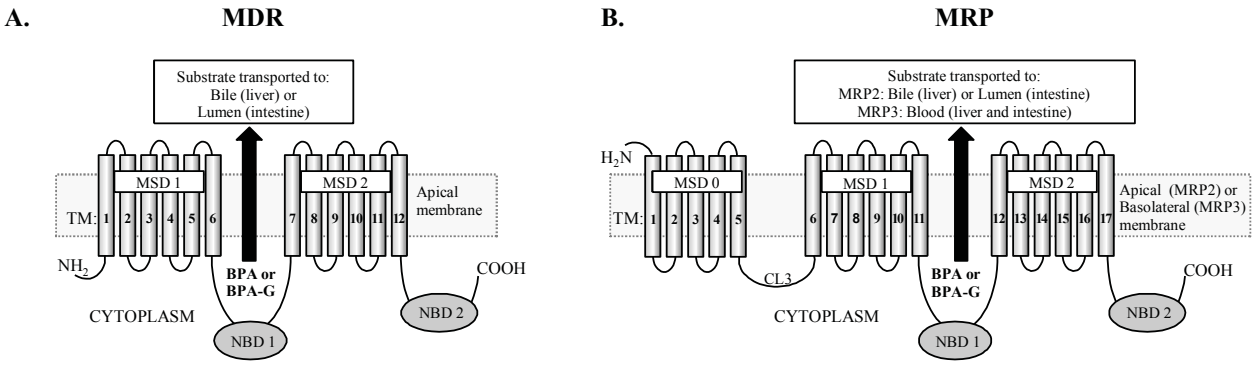


Figure 3

Figure 3. Interspecies comparison of human and rat ATPase activities of ABC efflux transporters with bisphenol A (BPA, 30 μM; A) and bisphenol A glucuronide (BPA-G, 30 μM; B).



MDR1 Region	Human MDR1	Rat mdr1a	Rat mdr1b	Reference
TM5	Ile306	Ile298	Val304	Loo *et al.,* 2003
TM6	Val338	Val330	Ile336	Loo *et al.,* 1994
TM6	Ile340	Ile332	Leu338	Aller *et al.,* 2009
TM6	Ala342	Ala334	Thr340	Loo *et al.,* 1994
TM8	Leu762	Leu754	Met759	Aller *et al.,* 2009
TM11	Ser943	Ser935	Ala940	Gros *et al.,* 1991
TM12	Leu975	Leu967	Met972	Hafkemeyer *et al.,* 1998
TM12	Gln990	Gln982	Asn987	Aller *et al.,* 2009

MRP Region	Human MRP2	Rat mrp2	Human MRP3	Reference
CL3	Ser195	Ser194	Glu209	Westlake *et al.,* 2003
CL3	Met246	Met245	Trp260	Ren *et al.,* 2001
CL3	Lys252	Lys251	Gln266	Ren *et al.,* 2001
TM6	Lys316	Lys312	Ala305	Haimeur *et al.,* 2004b
TM6	Lys344	Lys340	Ser333	Haimeur *et al.,* 2004b
TM11	Phe591	Phe587	Leu580	Campbell *et al.,* 2004
TM8	Val445	Val441	Pro434	Koike *et al.,* 2004
TM14	Pro1096	Pro1092	Ala1084	Koike *et al.,* 2004

Figure 4

Figure 4. MDR (A) and MRP (B) subfamily membrane topology and amino acid sequence differences in regions associated with substrate selectivity. Each transporter has multiple membrane spanning domains (MSDs) comprised of transmembrane (TM) α-helices. MSD1 and 2, along with their corresponding ATP nucleotide binding domains (NBDs), form the substrate binding cavity. MRP transporters have an extra MSD connected via a cytoplasmic linker (CL3) region. Species- and isoform-specific amino acid differences at positions known to mediate substrate specificity were identified (bold).

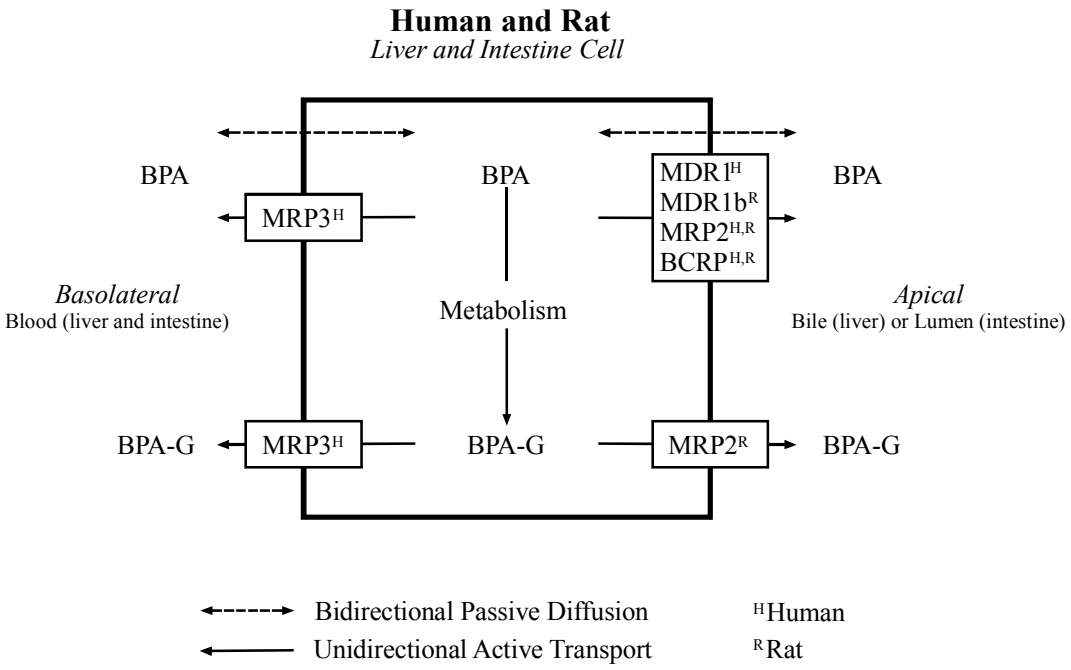


Figure 5

Figure 5. Schematic representation of the epithelial cell monolayer of the liver and intestine in both rat and human, which is composed of basolateral, cellular and apical compartments. Arrow in the cellular compartment denotes the metabolism of BPA to BPA-G. Active transport of the parent (BPA) and metabolite (BPA-G) at the apical and basolateral membranes are denoted by solid lines with single arrowhead. Bi-directional passive diffusion of BPA across the membranes is shown as dashed lines with double arrowheads. BPA and BPA-G specific transporters investigated in this study are shown in box and label format comparing human and rat orthologs.