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Determination of Amino Acid Requirements and Whole Body Protein Turnover in Two Strains of Broilers during Recovery from an Acute Phase Response

by

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Abstract

Lysine requirements, body weight changes and protein kinetics were determined in two strains of broilers, (commercial (C); random-bred (R)), during recovery from an acute phase response. In Chapter 2, LPS injection (I) decreased body weight gain of the C birds. The C birds had higher lysine requirements than the R birds.Requirements of the RI birds' were significantly higher than the un-injected (U) birds. In Chapter 3, LPS injection had no effect on body weight in either strain. The C birds had higher lysine requirements than the R birds and requirements were higher in the U birds than the I birds for both strains. The C birds had higher whole-body protein synthesis, breakdown, flux, and retention than the R birds. The I birds had significantly higher whole body protein breakdown than the U birds. These results suggest selection for growth may affect the innate immune response of broilers.

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List of Abbreviations

[ ] = Concentration

μ = Micro

ANOVA = Analysis of variance

BALT = Bronchus-associated lymphoid tissues

BCAA = Branched-chain amino acids

BW = Body weight

C = Commercial birds

CAT = Cationic amino acid transporters

CI = Commercial injected

Ci = Curie

CU = Commercial un-injected

DAAO = Direct amino acid oxidation

DNA = Deoxyribonucleic acid

DPM = Decays per minute

E. coli = *Escherichia coli*

FAO = Food and Agricultural Organization

g = Grams

GALT = Gut-associated lymphoid tissues

GLM = General linear model

HPLC = High performance liquid chromatography

IAAO = Indicator amino acid oxidation

IL = Interleukin

kg = Kilograms

L = Litres

LPS = Lipopolysaccharide

min = Minute

ml = Millilitres

NK = Natural killer cells

NO = Nitric Oxide

NRC = National Research Council

Oxmol = Moles of phenylalanine oxidized

PITC = phenylisothiocyanate

PAMP = Pathogen-associated molecular patterns

R = Random-bred birds

REC = Recovery

RI = Random-bred injected

RNA = Ribonucleic acid

rpm = Revolutions per minute

RU = Random-bred un-injected

S. typhimurium = *Salmonella typhimurium*

SRA = Specific radioactivity

TFA = trifluoroacetic acid

TNF = Tumor necrosis factor

UNU = United Nations University

vol = Volume

WHO = World Health Organization

Wt = Weight

1. Review of the Literature

1.1 Amino Acids

There are 20 known amino acids that can be used for protein synthesis (Table 1-1). All amino acids have a general structure consisting of a central carbon, an amine group, a carboxyl group and a characteristic side chain, R (Figure 1-1). Amino acids are often grouped chemically into three categories (acidic, basic or neutral) depending on the pH of their side chain (Table 1-1). However, nutritionally it is often more useful to group them by their status within an animal. Amino acids can be considered indispensable or dispensable depending on whether or not they can be synthesized by an animal (Leeson and Summers, 2001). A third category, conditionally indispensable amino acids, is given to those amino acids which become indispensable based on either an animal's physiological state (Reeds, 2000; Graber and Baker, 1973), or the quantity required to synthesize other amino acids (D'Mello and Lewis, 1970). The arrangement of the side chain around the central carbon also affects its bioavailability within an animal. All amino acids can be in either a D or L configuration with the exception of glycine as its side chain is hydrogen making it symmetrical regardless of configuration. The L isomer is the most commonly used in protein synthesis in higher vertebrates; however, methionine can be fed as either its D or L isomer in poultry (Katz and Baker, 1975) as it is able to be converted from the D to the L isomer (Baker and Boebel, 1980)

1.2 Amino Acids and Poultry Nutrition

Amino acids are an integral part of poultry nutrition as they provide the building blocks of protein. If one amino acid in the diet is limiting, it will restrict protein synthesis

to the level of that amino acid, and all other amino acids which are in excess will be oxidized. This has been an important concept to the industry as the limiting of an indispensable amino acid will not only restrict growth in poultry but also increase the cost to both the producer and the consumer (Si et al., 2001). In birds fed a cereal grain diet, methionine, lysine and threonine are often the first three limiting amino acids. Therefore requirements for these amino acids have been thoroughly examined in poultry (Leveille et al., 1960; Dobson et al., 1964; D'Mello, 1974; Han and Baker, 1994; Coleman et al., 2003). The dietary supplementation of synthetic amino acids started in the early 1950's with the commercial availability of D,L methionine (March et al. 1950). Protein composition of animal- and vegetable-based feedstuffs differ, and the amino acid profile of feeds provided to the animal does not always match exactly what is needed (Kircher and Pfefferle 2001).

With the addition of specific synthetic limiting amino acids to the diet, certain amino acid deficiencies can be avoided (Leibholz et al., 1986; Liu et al., 2007; Nahm, 2007; Wang et al. 2007). By providing amino acids as synthetic supplements to protein feedstuffs, the total amount of protein in the diet can also be reduced while still meeting the animal's requirements. This helps to limit the excess of other amino acids, some of which are otherwise deaminated and excreted as nitrogenous waste, while still providing those that are limiting in sufficient amounts (Creswell and Swick, 2001). However, the use of synthetic amino acids in the diet may cause unforeseen side effects as their utilization and absorption seems to differ from protein-bound amino acids (Williams and Duncan, 1980; Leibholz et al., 1986). Synthetic amino acids are considered to be 100% digestible, which is not usually the case with protein-bound amino acids (Williams and

Duncan, 1980; Leibholz et al., 1986). In addition, protein-bound amino acids are not always completely broken down and may be absorbed as peptides, thereby limiting the amount of microbial digestion and allowing more nutrients to go directly to the bird (Boorman and Ellis, 1996). Even with the use of synthetic amino acids given in the correct dietary proportions, bird performance on extremely low protein diets has been shown to suffer (Bregandahl et al. 2002; Fancher and Jenson, 1989). Therefore, crude protein in poultry diets must be included at a minimum value. While supplemented amino acids mabye 100% available, and their use may allow us to balance diets to reduce nitrogenous waste while still supplying adequate nutrition to the bird, the absorption of protein as peptides may be a required part of protein synthesis.

The efficiency with which amino acids are utilized also affects their accretion into body protein. Increasing this efficiency not only improves broiler growth and reduces costs by allowing a greater amount of amino acids to be available to the bird but also helps to reduce nitrogenous wastes produced by deamination of excess amino acids (Nahm, 2007). Several factors can reduce or increase the digestibility of amino acids such as feed processing (Wang and Parsons, 1998), diet type (Green et al, 1987), genetics (Lumpkins et al. 2007), and age of the animal (Huang, et al. 2005). A number of strategies have been examined to improve amino acid digestibility in birds including the use of exogenous enzymes, such as phytase (Peter and Baker, 2001; Rutherford et al. 2007), the addition of starches (Weurding et al. 2003), as well as improved feed processing techniques (Wang and Parsons, 1998).

As muscle is the main product of the broiler industry, we are continually trying to improve the performance of modern broilers through the manipulation of both diet and

genetics. The vast amount of literature on dietary amino acids and their efficiency of use for protein accretion in the bird emphasizes their importance in this area. However, some amino acids can be considered to have a more prominent role in muscle growth; one such amino acids is lysine.

1.3 Lysine in Broiler Diets

In poultry nutrition, the amino acid lysine is of particular importance, and being an indispensable amino acid the full requirement must be supplied through dietary ingredients. However, dietary grains, the primary ingredients in poultry feeds, tend to be limiting in lysine (NRC, 1994). Therefore the effects of a dietary lysine imbalance on the growth and performance in poultry have been well documented (Zimmerman et al., 1965; Tesseraud et al. 2001). There is a vast amount of literature which has addressed lysine requirements, not only because of its limiting status in many animal diets but because lysine is often used as a reference for the ideal protein concept. This concept refers to the profile of amino acids that exactly meets the animal's needs while minimizing the amount of amino acids used for energy or lost to nitrogen excretion (Firman and Boling, 1998). The theory behind this concept is that the ratio among the dietary amino acid requirements of an animal stays constant, at a similar age and metabolic state (Firman and Boling, 1998). This way, only the requirements for lysine must be determined for an animal; the requirements for all other amino acids can be calculated based on their ratio to lysine (Table 1-2). Lysine was chosen as a reference amino acid for a variety of reasons: analysis of lysine requirements are relatively straightforward compared to other amino acids, lysine is almost exclusively used for the accretion of protein, and in nearly

all monogastric livestock it is limiting in the majority of grain-based diets (Baker et al. 2002). As a result, past lysine research in poultry has primarily focused on balancing dietary lysine with the other indispensable amino acids to obtain optimal performance (Dobson et al. 1964, Baker and Han, 1994; Baker et al. 2002). In order to determine lysine requirements, dose-response trials are often used. These experiments involve feeding a diet in which one of the amino acids is supplied at graded levels above and below recommended level and the requirement of that amino acid is determined based on the level of dietary amino acid at which the growth response of the animal plateaus. (Barkley and Wallis, 2001). However dietary, environmental and genetic factors such as protein level, stocking density and sex (Baker, 1997), often complicate this process by changing amino acid requirements. (Mack et al. 1999). By using the ideal protein concept, even if absolute requirements for a single amino acid do change, the ratio between lysine and that amino acid should remain constant for a bird of the same age, genetics and metabolic state (Baker et al., 2002). The development of the ideal protein concept in swine lead to lysine becoming the gold standard for amino acid comparisons in both poultry and swine (Wang and Fuller, 1989).

1.4 Selection for Growth Rate

Modern-day poultry have been continuously selected for growth rate over the last several decades. It has been estimated that the growth rate of modern turkeys has doubled from 1966 to 2003 (Haverstein et al., 2007) and similar work in broilers has shown growth to have tripled from 1957 to 2001 (Havenstein et al., 2003) (Figure 1-2). However, this selection for growth rate may have unintentionally led to selection against several other important traits. As only a finite amount of resources are available to the

bird, heavy selection for one trait may cause resources to be preferentially allocated to the selected trait whereas less are available for other processes (Rauw et al., 1998). As a result of selection for growth, unfavourable physical characteristics, metabolic disease as well as reproduction problems have arisen in poultry. This has lead to increased consumer and producer concerns regarding animal welfare (Julian, 1998), productivity and mortality (Pakdel et al., 2005). In broilers, negative effects of selection for growth rate have been seen and addressed most noticeably in regards to mortality, as it has a considerable economic impact on the broiler industry (Pakdel et al., 2005). Ascites, a metabolic disease characterized by fluid accumulation in the body cavity and linked to fast growth rate in broilers, has increased over the last several decades as result of selection for growth (Julian, 1998). Reduction of growth rate using feed restriction has had the greatest effect on reducing the incidence of ascites (Camacho et al., 2004) however, the degree of feed restriction can lead to a decrease in both body weight and carcass yield which also leads to a capital loss for the poultry industry (Leeson et al., 1996; Acar et al., 1995). The incidence of skeletal deformities is also seen less often in slow-growing strains compared to those selected for rapid growth rate (Havenstein et al. 1994). Supplementation to the diet has been shown to have an effect on the incidence of tibial dischondroplasia (Leach and Lilburn, 1992), and angular bone deformities (McNamee et al., 1998; Julian, 1984), some of the more common physical disorders seen in poultry. Specifically supplementation of 1,25-$(OH)_2D_3$ has been shown to reduce the incidence of tibial dischondroplasia (Rennie and Whitehead, 1996) and may also be effective in reducing the incidence of rickets (Sanders and Edwards, 1991) by accelerating chondrocyte maturation (Farquharson et al. 1993). However like the ascites,

the physical deformities also seem to be reduced the most efficiently through the use of feed restriction. Elliot and Edwards (1994) showed that by withdrawing feed for several hours per day, several different strains of broiler chickens showed a reduction in the incidence of tibial dischondroplasia. It has also been established that reproductive viability is affected by selection for body weight gain. It has shown to result in decreases in a number of traits related to reproductive performance, such as fertility and sperm penetration, in both broiler breeders and turkeys (McGary et al., 2003; Hocking, 1993; Hocking, 1992; Arthur and Ablanalp, 1975). Recently, considerable work has gone into the selection and breeding of poultry to reduce these problems. For example, there has been successful selection for ascites-resistant lines (Druyan et al. 2007) as well as new information regarding the genes behind ascites susceptibility (Pakdel et al., 2005) making ascites less of a problem in breeding programs today then it has been in the past.

Considerably less work has been done on examining the impact of selection for growth rate on immunity. Whereas in turkeys some studies show no differences in either innate or adaptive immunity due to selection for growth (Cheema et al. 2007) in other studies, turkeys selected for growth experienced higher mortality rates when exposed to cholera (Saif et al., 1984) and lower antibody production in response to *Pastuerella multocida* and Newcastle Disease Virus (Sharaf et al., 1988; Nestor et al., 1996). Recent work has shown both innate and adaptive responses are affected by genetic selection for growth rate in broilers. Cheema et al. (2003) showed that 1957 random-bred broilers had an increased antibody response to sheep red blood cells compared to 2001 broilers. Conversely, when they looked at innate immune response, the 2001 broilers showed an increase in the number of sheep red blood cells phagocytized. Previous work in our

laboratory has shown that random-bred (since 1957 or 1977) broilers have an increased innate immune response when compared to modern-day broilers. Total bacteriocidal activity was higher in both random-bred strains, whereas oxidative burst of heterophils was greatest in the 1957 strain at most time points (0-20 min post stimulation; Saunders-Blades et al., 2006, unpublished data). This discrepancy between these two studies of whether selection for growth has increased or decreased the innate immune response could be the result of the different types of broilers used or could suggest that responses can be variable.

There has been work done looking at selecting concurrently for both growth and immunity. However, this selection has focused primarily on resistance to specific diseases such as Infectious Bursal Disease (Pitcovski et al., 2001) and Rous sarcoma virus (Pinard-van der Laan et al, 2004). In addition, although both traits are being selected for, this strategy often leads to birds with a lower growth potential than those purely selected for growth (Yunis et al., 2000).

Selection for growth rate has had unexpected effects on several other metabolic and physical processes. Understanding the changes that have occurred as a result of this selection will help the industry move towards correcting any adverse effects that have occurred. Therefore information on how selection for growth has affected the immune system will become a valuable asset to future selective breeding programs.

1.5 The Immune System

The immune system is comprised of two main branches, innate immunity and adaptive immunity. The innate immune system consists of cellular components, is present

in the bird from hatch, and is the first defensive reaction to either injury or pathogenic invasion. It is a rigid system, responding to only a limited number of stimuli including different structurally-conserved molecules found on invading pathogens (Holmskov et al. 1994). The adaptive immune response, like the innate system, is comprised of both humoral and cellular components and is an important mechanism for destruction of and memory of antigens encountered by the innate system. The adaptive system can constantly change depending on the antigen it is facing. The innate system is an integral part of this response as it allows the adaptive system to differentiate between substances that require an immune response and those that do not (eg. self and non-infectious agents) (Fearon and Locksley, 1996). In commercial situations, poultry are constantly exposed to microbes from various sources in their environment (Apajalahti et al., 2004). These sources include the air, the water, the feed and the litter (Humphrey and Klasing, 2004). The innate immune system is especially important in young poultry as the adaptive system can take up to a week to mature post hatch (Wells et al. 1998) and it provides the bird with a first line of defence against these pathogens.

The components of the innate system include physical barriers such as the skin, mucus membranes and bodily secretions as well as a variety of lymphocytes, chemical messages, and defensive mechanisms. These serve to stop or destroy the invading pathogen and stimulate an adaptive response. If the physical barriers are penetrated, both cellular and humoral mechanisms are involved in seeking out and destroying infectious agents and repairing tissue damage (Powell, 1987). Once stimulated, a variety of innate immune cells mobilize to destroy the invading pathogen and release chemotactic signals (cytokines) to stimulate initiation of the complement cascade and acute phase protein

production as well as activate the adaptive immune system (Cruse and Lewis, 1999). A number of non-specific pathogen associate molecular patterns (PAMP) are used by immune cells to identify foreign material and stimulate the innate system. Examples of common PAMP include gram-negative bacterial lipopolysaccharide (LPS), gram positive bacterial lipoteichoic acid, bacterial flagella, double-stranded RNA commonly found in viruses, mannose, N-formylmethionine, fungal glucans and petidoglycan (Ingle et al., 2006). Inflammation is one of the first responses to tissue damage and infectious agents. Inflammation results in the migration of lymphocytes to the site of infection as well as the dilation of blood vessels and increased tissue permeability to the immune cells (Baumann and Gauldie, 1994; Suffredini et al., 1999; van Miert, 1995). Lymphocytes involved in innate immunity include macrophages, heterophils, natural killer cells, thrombocytes and dendritic cells. In general these innate immune cells work to recognize and destroy potential pathogens as well as to stimulate antibody production and other parts of the acquired system (Fearon and Locksley, 1996).

1.5.1 Natural Killer Cells

Natural killer (NK) cells play a critical role in the host's defense against viruses (Biron et al. 1999) as well as contributing to the defense against invading bacteria and parasites (Scharton-Kersten and Sher, 1997). A major role of the NK cell in humans, and one that has received a great deal of research, is their ability to differentiate between healthy and damaged cells using a variety of complex mechanisms making them key in identifying and terminating infected or mutated self cells (Magor and Magor, 2001). An increase in the function of NK cells has been shown to reduce the effects of Marek's disease and Newcastle virus in poultry (Jarosinski et al. 2001; Marsh et al. 2001). This

could be important when considering that activity of NK cells in poultry have been found to be inhibited by the stress caused by common activities such as physical restraint (Kushima et al., 2003).

1.5.2 Dendritic Cells

In mammals, dendrititic cells originate in the bone marrow as lymphoid cells and migrate to the skin and lymphoid organs such as the spleen and the thymus (Brocker et al. 1997). They act as antigen presenting cells, are involved in the induction of further immune responses as well as help to eliminate self-reactive T-cells (Brocker et al. 1997). Their role in poultry is less well defined. Although Langerhan-type cells (dendritic cells of the skin) have been identified in the epidermis of the chicken (Igyártó et al. 2006), their function has not yet been clearly characterized.

1.5.3 Thrombocytes

Thrombocytes are the analogue of human platelets; they are involved in the process of hemostasis (Stalsberg and Prydz, 1963), working to cause aggregation and start the formation of clots (Ronald and Birrenkott, 1998) and are considered a highly phagocytic cell type in chickens (Chang and Hamilton, 1979). Chicken thrombocytes display a shift in morphology when the bird is stressed, this change in morphology can be used as an indicator of stress (Gross, 1989) similar to the use of the heterophil:lymphocyte ratio (Gross and Siegel, 1983). This could be a useful observation as hormones, such as catecholomines produced in stressful situations, (Beuving and Blokhius, 1997) can impact the ability of the immune system to cope with pathogens by depressing both cellular (Regnier and Kelly, 1981) and humoral immunity (Henken et al., 1983)

1.5.4 Heterophils

Heterophils are phagocytic cells involved in avian innate immunity. Although several differences have been shown to exist between the two cell types (Montali, 1988), heterophils are essentially the avian counterparts to mammalian neutrophils (see Harmon, 1998 for a review; He et al. 2005). Heterophils are one of the earliest cells to respond to an immune challenge, when mobilized by cytokines such as immune lymphokines derived from T-cells (Kogut et al. 1998) they can mount an effective immune response within 30 min (Kogut et al. 1994). Invading pathogens are ingested by heterophils through phagocytocis, and both oxygen intermediates and proteolytic enzymes are thought to play a role in bacterial destruction (Desmidt et al., 1996; Genovese et al., 2000; Kogut et al., 2001; Swaggerty et al. 2003). Although heterophils, like neutrophils, produce an oxidative response, this does not seem to be their main form of bactericidal activity as it is considerably less effective than that found in mammalian neutrophils (Harmon, 1998). In addition myeloperoxidase, a lysosomal protein involved in the oxidative destruction of bacteria and stored in the granules of neutrophils, is thought not to be present in heterophils (Penniall and Spitznagel, 1975) however more recent work has suggested that low levels of a myeloperoxidase-like protein may be present (Lam, 1997). It has been hypothesized that avian heterophils rely on other methods, such as degranulation, the discharge of granules into the vacuole, to kill phagocytised bacteria (Stabler et al. 1994). Heterophils are an important part of the primary immune defence in the avian lung, as heterophils reside in the associated lymphoid tissues (Reese et al. 2006), whereas macrophages must be mobilized (Toth and Siegel, 1986 ; Reese et al.,

2006). Therefore, they become an important mediator of resistance to many inhaled airborne pathogens.

1.5.5 Macrophages

Macrophages are recognized for their role as scavenger cells in the immune system, as one of their major functions include the phagocytosis and destruction of foreign material via lysosomal enzymes (Sun-Wada et al. 2003). Phagocytosis can be induced by one of three processes: non-specific activation, specific activation or by components of the complement system, it is one of the key systems involved in bacterial destruction (Powell, 1987). In addition to this, macrophages play a role in alerting the adaptive immune system to the presence of foreign antigens, following phagocytocis, by presenting these antigens to T and B cells. As macrophages are one of the most widely-dispersed immune cells in the body, they are ideally suited for their role as regulators of the immune response (Klasing, 1998). Depending on their location, macrophages can be classified by a variety of different names including osteoclasts in the bone; aleveolar cells in the lungs; histiocytes in the connective tissue; Kupffer cells in the liver; microglial cells in the neural tissue; and sinusoidal cells in the spleen. Regulation of type and intensity of an immune reaction is controlled through the release of a variety of chemical signals such as cytokines (Klasing, 1998), cytokine inhibitors (Calandera et al. 1994), hormones (Hendricks and Mashaly, 1998), as well as reactive nitrogen (Sung et al. 1991) and reactive oxygen species (Golemboski 1990). Macrophages originate from the bone marrow and circulate in the blood stream as immature monocytes until they migrate into the tissues where they become mature macrophages (Powell, 1987). Migration to a tissue

is caused by chemotaxis which can be initiated by a variety of stimuli including tissue damage, cytokines or the pathogens themselves.

In poultry, the response of macrophages has been extensively studied in a variety of diseases (Okamura et al. 2005; Olkowski et al., 2005). In coccidiosis, a parasitic infection of the intestine caused by *Eimeria* (Dalloul and Lillehoj, 2005), cytokine and chemokine expression have been shown to differ depending on the species of *Eimeria* to which the bird is exposed (Dalloul et al., 2007). In *Salmonella* infections, the macrophage phagocytic response is dependent on time of exposure and genetic potential for growth of the bird (Van Hemert et al., 2007). Early in infection, fast-growing lines of broilers seemed to have greater phagocytic activity than slow-growing lines, whereas 5 days post-infection, the slow-growing line showed a greater phagocytic response than the fast-growing line. In cellulitus, a skin condition caused by *E. coli* (Tessier et al., 2001), fast-growing broiler chickens have fewer phagocytic cells, such as macrophages, than slow-growing leghorn chickens, possibly making broilers more susceptible to the disease (Olkowski et al., 2005).

1.5.6 The Acute Phase Response

The acute phase response consists of a series of inflammatory reactions that result in a range of defence mechanisms including fever, increased passage of white blood cells to infected tissues, the release of pro-inflammatory cytokines and vasodilation (Baumann and Gauldie, 1994; Suffredini et al., 1999). Initiation of the acute phase response often causes undesirable effects on production, including impaired reproduction (Parker et al., 2002), decreased bone strength (Mireles et al., 2005), anorexia, and breakdown of skeletal muscle (Klasing et al., 1987; Barnes et al., 2002). Once the acute

phase response is initiated, the liver alters its function in order to synthesize large amounts of acute phase proteins to mediate the inflammatory process (Tilg et al. 1997). Nutrients, especially amino acids, are required in order to support the production of the acute phase proteins and are also used in small quantities in order to form mediator molecules such as cytokines and replicate immune cells (Klasing, 1998). Skeletal muscle, the most economically important tissue of the broiler industry, is the largest quantity of labile amino acids in the body; and is preferentially broken down in order to provide nutrients and energy to the immune system (Hentges et al. 1984).

Three main groups of cytokines are released during the acute phase response: those that regulate growth of cells, the pro-inflammatory cytokines and the anti-inflammatory cytokines (van Miert, 1995). Pro-inflammatory cytokines initiate the acute phase response, resulting in muscle catabolism, acute phase protein production and anorexia. Specifically TNF-α and IL-1 result in the catabolism of muscle and an increase in the overall body amino acid turnover respectively (Gruys et al. 2005). Initiation of the acute phase response and production of acute phase proteins in the liver are initiated by IL-1-like and IL-6-like cytokines in birds (Moshage, 1997). Acute phase proteins produced can be positive or negative, depending on whether they increase or decrease, respectively, during the acute phase response (Gruys et al. 2005). From an experimental and clinical point of view, the appearance or disappearance of acute phase proteins in the blood can indicate the presence of sub-clinical infections. As well, measuring changes in acute phase proteins provides the ability to monitor changes in the acute phase response over time (Chamanza et al. 1999). In veterinary medicine, measurements of acute phase proteins have been used to detect the appearance of an acute phase response in a number

of different animals (Peterson et al. 2004). Currently acute phase proteins are being used to monitor disease in humans (Ferard et al. 2002), poultry (Holt and Gast, 2002) and cattle (Saini et al. 1998), to assess post-surgery recovery, onset of infection, and potential food safety issues, respectively. Much more is known about acute phase proteins in mammals than birds and differences in acute phase proteins between the two groups are only starting to be defined.

1.5.7 Associated Lymphoid Tissue in the Bird

Unlike mammals, birds do not have a functional lymphatic system. Instead, they rely more heavily on several aggregates of organized lymphoid tissue to protect vulnerable tissues from infection. These areas include both the gut and the lungs; the lymphoid tissues present here are known as the gut-associated lymphoid tissues (GALT) and the bronchus-associated lymphoid tissues (BALT), respectively.

1.5.7.1 GALT

As the gut essentially acts as a barrier between the bird and its environment, it is constantly at risk from both mechanical injury and pathogenic invasion. As such, the gastrointestinal tract is a primary location of both innate and adaptive immune defences in the bird. The bursa of Fabricuius, a primary organ in the adaptive immunity of the chick, is a blind sac located on the dorsal part of the cloaca (Glick et al, 1956) and is involved in the production of B-cells, which are responsible for antibody production (Ratcliffe et al., 1996). If chicks are bursectomized or the bursa is subjected to disease at an early age, antibody production is severely suppressed (Mueller et al,. 1960; Allan et al., 1972). As a result, any disease which affects the development of the bursa of Fabricuius could have significant effects on immune competency of chicks. Peyer's

patches, a second tissue involved in gut immunity, are found in several sections of the intestine in young chicks with only one Peyer's patch being dominant in adult birds (Schat and Myers, 1991). Peyer's patches contain microfold cells which are involved in presenting antigens in the intestine to underlying T and B cells (Muir, 1998). Other tissues such as the cecal tonsils, cells in the lamina propria and epithelial lining have similar roles in the immunity of the intestine; macrophages and/or lymphocytes can also be found dispersed within each of these tissues (Schat and Myers, 1991). Meckel's diverticulum is the remainder of the yolk sac, and contains populations of both macrophages and lymphocytes, as well as T and B cells, yet the function of this tissue in immunity is as yet unknown (Muir, 1998).

1.5.7.2 BALT

Like the gut, the lung is a barrier between the outer and the inner environments of the bird. Unlike the gut, which has several distinct lymphoid structures, BALT seems to mainly consist of diffuse lymphocytes scattered within altered epithelial tissues in Peyer's patch-like nodules (Bienenstock et al, 1973). However, research on BALT in poultry is limited. Morphology as well as content of the immune tissue, has been shown to change with both age and environment (Jeurissen et al. 1994). Inhaled particles are cleared in the lungs by the epithelium and interstitial macrophages (Nganpiep and Maina, 2002). As an increase in the number of particles in the air may cause subclinical immune responses by constantly activating these defences, by limiting the amount of debris in the air, it may be possible to improve bird growth, similar to the observations of Cook and Bird (1973) when examining GALT in germ-free chicks. However, to date research on

the BALT in birds in germ-free environments has focused on formation of BALT itself rather than the growth of the bird (Bang and Bang, 1968).

1.6 Antibiotic Alternatives in Poultry

Currently in North America, several prominent diseases in poultry are controlled through the addition of additives and prophylactic doses of antibiotics to the feed. However as consumer perceptions about antibiotic use in animal industries change and concerns about antibiotic resistance increase, alternative methods of keeping birds healthy and productive need to be examined. Recently, several methods have been investigated in order to improve poultry resistance to disease without the therapeutic use of antibiotics (Parks et al. 2001; Huff et al., 2002 Guo et al. 2004). These methods include the use of bacteriophages (Sklar and Joerger, 2001), egg yolk antibodies (Malik et al. 2006), competitive exclusion (Starvic and D'Aoust, 1993; Wooley et al. 1999), antimicrobial peptides (Evans et al. 1995), probiotics (Corrier et al. 1995), and cytokines (Lowenthal et al. 1999) . Probiotics, the feeding of supplemental live microbial species in order change the intestinal flora, has been used in attempts to control necrotic enteritis (for review see Van Immerseel et al., 2004). Although it has been shown to successfully reduce the number lesions seen in poultry (Hofacre et al., 1998), it seems to be less effective than competitive exclusion, shifting the chicks microflora over a short period of time using other competitive microbes (Hofacre et al., 1998). Lymphokines, a type of cytokine, has been shown to enhance the function of heterophils in newly hatched chicks (Genovese et al., 1999) and up to 5 days post-hatch (Genovese et al. 2000) without causing an adverse immune response (Kogut et al. 1996). However, there have been

several problems associated with the methods mentioned previously including the potential for microbes to develop resistance against antimicrobial peptides or bacteriphages (Peschel and Colins, 2001; Joerger, 2003); manipulation of the bacterial genome by the agent to produce a more virulent strain of bacteriophages (Figueroa-Bossi and Bossi, 1999); or reduced effectiveness and stability of many of these treatments when administered by common routes (Joerger, 2003). Proper management of animals has been shown to be an effective way to maintain growth and productivity. A clean environment has been shown to reduce the affect antibiotics have on poultry. In addition, chicks raised in a germ-free environment have been shown to grow more quickly than chicks raised in dirty environments (Cook and Bird, 1973) despite a reduction in the amount of intestinal wall tissue. This may in part be due to both lowered maintenance requirements of the chick as well a reduction in the subclinical immune response caused by constant exposure to microbes.

1.7 The Effect of Dietary Amino Acids on Immunity

Nutritional modulation of the immune system has been investigated on many occasions (Zhang et al. 2005; Wang et al., 2004, Kwak et al. 1999). Amino acids, especially arginine, glutamine, lysine and the branched-chain amino acids (BCAA) have been a particular focus of nutritional immunity research in both humans and animals. Amino acid deficiencies during an immune response have been shown to depress lymphoid organ weights, (Konashi et al. 2000), IL-1 response, feed intake (Klasing and Barnes, 1988), and antibody production (Tsiagbe et al. 1987).

Branched-chain amino acids include isoleucine, leucine and valine, each of which are indispensable, neutral amino acids for both mammals and birds. The use of BCAA during recovery from illness in humans has been thoroughly examined in the medical field. In humans, supplemental BCAA have been shown to aid in muscle recovery following damage (Shimomura et al, 2006). This suggests potential benefits in relation to muscle recovery after an immune response in poultry. High levels of BCAA have been shown to be well-tolerated in variety of species (Baker, 2005) suggesting that they could be included as a supplement in the diet. However the valine requirements during an immune response have been shown not to differ from that for growth in 3 to 6 week old broilers (Thornton et al. 2006). In addition, deficiencies in isoleucine do not seem to affect immune responses of 30- to 42-d-old broiler females to a great extent (Hale et al. 2004). This could suggest that leucine rather than valine or isoleucine may be responsible for the benefits seen with supplemental BCAA or that these benefits may be age-dependant. The role of BCAA in the poultry immunity is unclear, and needs to be more thoroughly examined before their importance in maintaining performance and health can be determined.

Glutamine has received much attention in the area of immunity in both animal and human nutrition. Glutamine is an acidic, dispensable amino acid which is involved in a variety of metabolic processes including protein synthesis, nitrogen metabolism, formation of other dispensable amino acids, and the citric acid cycle (Neu et al. 1996). Glutamine metabolism provides a large amount of energy to macrophages and lymphocytes and provides nitrogen for purine and pyrimidine synthesis (Ardawi and Newsholme, 1983), nucleotide bases used in the production of DNA. A decrease in

plasma glutamine concentration is often associated with many types of immune challenges including burns, surgery and physical trauma (Walsh et al., 1998). Glutamine is often utilized in large amounts by the immune system, as an energy source for lymphocytes and macrophages as well as nitrogen and carbon precursors for RNA/DNA synthesis, and regulating replication of leuckocytes and other immune cells. Therefore, a fall in glutamine concentration can often result in impaired immune function (Brambilla et al., 1970) and is characterized by a decrease in leukocyte and cytokine formation (Parry-Billings et al., 1992). Supplementation of glutamine increased lymphocyte response and leukocyte counts in piglets infected with *E. coli* (Yoo et al., 1997). Oral glutamine supplementation to hospitalized patients helped to increase gut barrier function against opportunistic pathogens (Wischmeyer, 2003). This was most likely a result of glutamine, as well as the amino acid glutamate, being preferred energy substrates of the gut epithelium (Watford et al. 1979); this includes cells involved in gut immunity such as goblet cells and Paneth cells (Dibner and Richards, 2004). Therefore glutamine has been shown to be an important part of gut-based as well as overall immunity.

Arginine is classified as a basic, indispensable amino acid in birds and has been linked to improved immunity, especially in birds. As birds are a uricotelic species, unlike mammals, they do not possess a complete urea cycle (Tamir and Ratner, 1963). Birds in particular lack the enzyme carbamoyl phosphate synthetase, which combines ammonia and bicarbonate to make carbamoyl phosphate (Tamir and Ratner, 1963). Arginine itself is synthesized as part of the urea cycle, making it dispensable or conditionally dispensable in mammals depending on their physiological state (Gharbi et al., 2005). The lack of a complete urea cycle makes poultry unique in the livestock industry as they need

the majority of their arginine requirement provided in their diet. As a result, a great deal of research has gone into examining arginine's role in both growth and immunity in poultry. Arginine's main role in the immune system is as one of the major substrates for nitric oxide (NO) production (Collier and Vallence 1999). Nitric oxide is a reactive nitrogen species used by macrophages to neutralize foreign microbes (Sung et al 1991) in response to cytokines, acts to increase vasodialation to the site of injury or infection, and helps bind leukocytes to the endothelial wall (Tong and Barbul, 2004). Supplemental arginine has been shown to increase survival from tumors (Taylor et al 1992), improve vascular health (Wideman et al., 1995); increase the weight of the spleen and thymus, which are important organs in immunity (Kwak et al., 1999); increase the percentage of CD8+ cells, a type of cytotoxic T cell, which represents an increase in general host immune competence (Lee et al, 2002); and increase the heterophil:lymphocyte ratio during a viral infection (Lee et al, 2002) suggesting an increase in early phagocytosis (Boa-Amponsem, 2000).

Another amino acid which may play an important role in immunity is lysine. Like arginine, lysine is also a basic, indispensable amino acid in chickens. Whereas the effects of lysine on growth have been extensively studied in poultry (Hill and Olsen, 1963; Zimmerman and Scott, 1965; Vazquez et al., 1997; Kidd et al., 1998) compared to arginine and glutamine, little work has been done to determine the role of lysine in the immune system. Lysine is important in growth as it is often the first or second limiting amino acid in grain-based diets for poultry (National Research Council (NRC), 1994). Due to each amino acid being required in specific amounts for protein synthesis, if lysine is limiting, protein synthesis is restricted to a sub-optimal rate. During an inflammatory

response certain tissues may be vulnerable to lysine deficiencies (Humphrey et al. 2006). This vulnerability may result from metabolic regulation of cationic amino acid transporters (CAT 1, 2 and 3) in order to protect tissues important to immunity from deficiencies (Humphrey and Klasing, 2004; Humphery and Klasing 2005). These transporters are involved in the transport of lysine and arginine to and from tissues and their affinity changes depending on the physiological state of the bird (Humphrey et al., 2006). During an immune response, up-regulation of these transporters has been seen in the bursa and the liver whereas down-regulation is seen in the thymus (Humphrey et al., 2006). This down-regulation in transport makes the thymus potentially vulnerable to lysine deficiency (Humphrey and Klasing 2004). This may be important in relation to the amount of lysine currently supplied in broiler diets. Lysine is supplemented for optimal growth, and during an immune response lysine is preferentially used by the immune system. Consequently, depending on the needs of the immune system and the extent of down-regulation in other tissues, the current dietary lysine recommendations for growth may not be optimal for immunity and the needs of down-regulated tissues during an immune response. However, Klasing and Barnes (1988) found that the needs of broiler chicks for lysine under immunological stress are less than for growth under normal growing conditions, suggesting this may not be the case. Deficiencies in lysine during an immune challenge have been shown to suppress certain immune responses. Lysine deficiencies in growing chicks challenged by Newcastle disease virus not only reduced the final body weight of the birds, but also the relative size of the spleen, a reservoir and filter of blood (Chen et al., 2003). Chen et al. (2003) also discovered that antibody response to Newcastle disease was lower in birds fed inadequate levels of lysine

compared to those fed adequate diets. Therefore, the needs of the poulty immune system for the aforementioned amino acids should be quantified in order to determine if the current amount supplied for growth are also optimum for immunity.

1.8 Protein Turnover in Broilers

The accretion of dietary protein into skeletal muscle tissue is the basis of the commercial broiler chicken industry. Body protein deposition increases as balanced crude protein in the diet increases up to the animal's requirement. Past the requirement, although protein synthesis still increases as dietary protein is increased, protein breakdown also increases at a linear rate (Urdaneta-Rincon and Leeson, 2004). Maruyama et al. (1978) found that in birds with variable growth rate, rates of protein turnover were more affected by the decrease in protein degradation than the increase in protein synthesis in 2-week-old chicks. When protein turnover was examined in protein-deprived rats, the restoration of protein to the diet caused an immediate reduction in protein breakdown with an eventual increase in protein accretion (Young et al. 1971). However, this point is not unanimously agreed upon as several researchers have found that protein synthesis plays more of a role in net protein turnover in both broiler chickens (MacDonald and Swick, 1981) and rats (Millward et al. 1975) with little change in the breakdown. Rates of protein turnover have also been shown to differ in poultry with differing rates of growth. When comparing lines of broilers divergently selected for growth rate, it was found that fractional degradation rates were significantly lower in the breast muscle of the faster growing birds (Tesseraud et al. 2000). In addition, in broilers selected for leanness, the rate of protein deposition was higher than in birds with higher amounts of body fat (Macleod et al., 1988; Pym et al., 2004). This trend is also true in

broilers selected for higher breast meat yield (Pym et al., 2004). The question as to whether degradation or synthesis plays a bigger role in protein accretion and its answer are important in terms of bird growth as their manipulation may allow an increase in the efficiency of muscle accretion.

During an immune response, organs and cells play a large role in the whole body protein turnover of the bird. During an acute phase response, the liver produces large amounts of acute phase proteins (Waterlow, 1984) from dietary amino acids as well as from muscle breakdown (Reeds et al., 1994). However, it is theorized that the composition of the muscle protein does not closely match that of the newly-synthesized acute phase proteins (Reeds et al., 1994). This, in addition to anorexia, is what is thought to result in a large amount of muscle protein breakdown. This is largely due to the increased need of the liver for amino acids such as phenylalanine, tyrosine and tryptophan, as these amino acids are found in smaller quantities in skeletal muscle than in acute phase proteins (Reeds et al., 1994). A greater amount of protein is then mobilized from skeletal muscle than is produced in the form of acute phase proteins. As a result, a major immune challenge can often result in economic losses as the amount of breast muscle deposition is decreased. These losses can be quantified in the form of bird mortality, cost of the treatment or smaller final body weights (Zhu and Hester, 2000).

Causing an even greater economic loss to the industry may be sub-clinical infections. These challenges may not cause immediate changes in production, however they can still result in inflammation (Apajalahti et al., 2004). This constant stimulation can cause a small but continuous redirection of nutrients from growth to the immune system, ultimately limiting the growth of the bird (Cook and Bird, 1973). The constant

stimulation of the immune system due to pathogens in the environment has the potential to mobilize a great deal of muscle protein throughout a bird's lifetime. When examined on an industry scale, this could result in a substantial economic loss. Measuring protein turnover in a variety of environments and physiological states will help to provide insight into not only rates of degradation and synthesis but also into the effect of both sub-clinical and acute immune responses on protein accretion. From the perspective of poultry production, knowing how protein turnover is affected in a variety of situations will help to increase our understanding of protein accretion during an immune response and how selection for growth rate has changed this process

1.9 Measuring Protein Turnover

There are several different levels at which protein turnover can be measured, these levels include at a whole body level or at the level of a single tissue or in specific proteins. There are two commonly used methods to measure whole-body protein turnover: the precursor method and the end product method. The precursor method is more widely used than the end product method (Dugleby and Waterlow, 2005) and is often considered the standard in determining whole body protein turnover in humans. The precursor method involves the constant infusion of a labelled amino acid such as [1-^{13}C]- leucine followed by measurement of enrichment in both the blood and the breath (Wagenmakers, 1999). Whole-body protein turnover in poultry has been compared to mammalian protein turnover and on a g/kg of body weight basis poultry protein turnover tends to be double that of several mammalian species such as the pig, rat and man (Reeds and Lobely, 1980; Muramatsu and Okamura, 1985; Muramatsu et al., 1987; Hiramoto,

1989). This difference has been attributed to the higher body temperature of avian species compared to mammals, as this would increase the elongation rate of the polypeptides in the muscle by approximately 35% which would in turn increase the RNA activity of the muscle, increasing protein turnover (Laurent et al., 1978).

Although the analysis of whole-body protein turnover allows for broad comparisons of changing trends in protein turnover, information on the breakdown and synthesis within single tissues can sometimes be more valuable. This is especially true in poultry where skeletal muscle tissue and its turnover hold high economical significance. The measurement of 3-methyl histidine has been widely used in a number of species to measure protein breakdown in muscle (Young et al. 1972; Long et al. 1975; Harris and Milne, 1981; and Hayashi et al. 1985). This technique was established in order to get around problems with amino acid recycling seen in earlier tracer decay experiments when measuring amino acid breakdown in muscle (Millward, 1970). This method has been extensively studied and it is widely believed that 3-methyl histidine is only found in actin/myosin and excreted without being reutilized for protein synthesis (Saunderson and Leslie, 1988). However there is research showing that 3-methyl histidine can be released from other tissues such as the intestine (Harris and Milne, 1981) and therefore the question as to whether this method is accurate enough to determine protein breakdown from only skeletal muscle remains.

Two of the more extensively used methods of determining protein synthesis are the flooding method and the constant infusion method (Garlick et al. 1973; Garlick et al. 1980, Muramatsu and Okumura, 1985). Both methods involve the infusion of a labelled amino acid followed by measurement of that amino acid or its metabolites in specific

tissues (Garlick and McNurlan., 1998). The difference between them is that the constant infusion technique involves the constant infusion of the amino acid whereas the flooding dose method floods the system with a labelled amino acid contained within a larger unlabeled bolus of the same amino acid thereby equalizing the labelling in the tissues and the plasma (Garlick et al. 1994). Several problems have been identified with the constant infusion method. The long time in which it takes amino acids to reach a plateau in the plasma can lead to either a decrease in protein synthesis (Preedy et al., 1984) or inadequacy to properly portray the normal state if meals are given constantly (Garlick et al. 1994). In addition this method can cause the added problem of the free amino acids in the tissue having a lower radioactivity than the plasma as amino acids are constantly released from the surrounding tissue (McNurlan et al. 1991). This method therefore does not always accurately reflect rates of protein turnover in the body. The flooding dose method eliminates these problems by equalizing the incorporation of the labelled amino acids into both the tissues and the plasma. However, whether or not the excess amino acids affect the normal course of protein turnover has been questioned (Pomposelli et. al., 1985) and is still under debate. Whereas Garlick et. al. (1980) found no difference in fractional protein synthesis rates when rats were given a flooding dose compared to non-flooded controls and Lobley et. al. (1992) found similar results in sheep using both methods, Pomposelli et. al. (1985) found differences in results in the liver, muscle and whole body of rats.

1.10 Determining Amino Acid Requirements

As body protein is composed of the 20 different amino acids, and as protein turnover, and therefore bird growth, is directly affected by the amount of limiting amino acids in the diet, methods to determine amino acid requirements have been thoroughly examined. Several different methods have been utilized over the years to determine amino acid requirements in both animals and humans. These methods include the use of nitrogen balance and growth, direct amino acid oxidation (DAAO), Indicator amino acid oxidation (IAAO), and 24-h amino acid balance. Nitrogen balance and growth are considered to be the standards for determining amino acid requirements and have been the most widely used in many species (Lellis and Speer, 1987; Protho, 1989; Samadi and Liebert, 2007). Many of the human requirements for indispensible amino acids are based on this method (FAO/WHO/UNU, 1985). The nitrogen balance technique involves the feeding of a complete diet, except for the amino acid under investigation, which is provided in graded quantities ranging from deficient to excess. Excreta is collected over a 5 – 10 day period (Tzeng and Davis, 1980; Samadi and Liebert, 2007); following this, the requirement for the limiting amino acid is calculated based on the retention of nitrogen in the body, its excretion, and the balance between the two. There is an ongoing debate over whether this technique is accurate in determining amino acid requirements, as many of the more modern methods estimate higher requirements for many amino acids (Young, 1992; Brunton et al., 1998). However, it has been speculated that this variation may be in part due to a lack of knowledge of the dynamics of protein metabolism (Millward and Rivers, 1986) or to incomplete recovery of excreted nitrogen coupled with experimental error (Fuller and Garlick, 1994).

Methods developed as a substitute to the nitrogen balance method included DAAO, 24-h amino acid balance studies and more recently, IAAO. DAAO involves feeding a graded level of a radio-labelled test amino acid in the diet and measuring the oxidation rate of that amino acid as the dietary levels increases. At deficient levels of intake, a low and constant oxidation rate will occur, as most of the test amino acid is used for protein synthesis. At excess levels of the test amino acid in the diet, only a portion of that amino acid will be used for protein synthesis, as other amino acids will be limiting, and the rest of the test amino acid will be oxidized. This will cause a steady linear increase in the oxidation rate of the amino acid as dietary levels increase (Figure 1-3; adapted from Pencharz and Ball, 2003). Whether this method is a true reflection of amino acid requirements has been questioned by Millards and Rivers (1986; 1988) as it is believed to over-estimate requirements. They argue that adaptation to a prior level of that amino acid may affect the requirement as well as that the tracer will become diluted as intake of the amino acid increases, leading to an overestimation of true requirements. The duration of the oxidation has also been questioned. It has been felt that the 4 to 8 hr duration of the studies do not truly reflect amino acid requirements, as measurements are only taken over a short period of time when the test subject is in a fed state (Carpenter, 1992). However, 24 hr amino acid balance studies showed results similar to the shorter direct oxidations studies taken in a fed state (El-Khoury et. al., 1994). Although this study alleviated the problem of whether duration of oxidation affected requirements, the question of adaptation and tracer dilution still remains. These limitations were recognized and led to the validation of IAAO, a quicker, more efficient way of determining amino acid requirements that avoids some of the problems associated with DAAO, as it uses the

oxidation of a separate amino acid, not the one under investigation, as an indicator of the amino acid under study (Zello et al., 1995).

1.11 The IAAO Method

The IAAO method involves the use of two different amino acids with separate oxidative pathways, the test amino acid and the indicator amino acid. The test amino acid is an indispensable or conditionally dispensable amino acid and is delivered to the bird via the diet. Individual birds receive varying concentrations of the test amino acid in an otherwise nutritionally-complete diet, ranging from extremely above to below the expected requirement. The indicator amino acid is radioactively labelled and delivered to the bird either as a primed oral dose every half hour (Leslie et al., 2006) or as a venous infusion (Coleman et al. 2003) during the experiment. The birds are placed in individual oxidation chambers with a known flow rate of air and the CO_2 which is exhaled by each bird is collected. A known proportion of the collected CO_2 is then analysed for labelled carbon. These data can then be used to determine the amount of labelled amino acid which was oxidized by birds at each of the test amino acid concentrations. Determination of requirements using the IAAO method uses the limiting amino acid concept. When the test amino acid is fed below requirement it becomes limiting to protein synthesis causing the indicator amino acid to be in excess. As the test amino acid in the diet increases above the requirement it is no longer limiting and the indicator amino acid is oxidized at a constant rate. When plotted against the concentration of the test amino acid in the diet, a constant decrease in the oxidation of the indicator amino acid is observed until a plateau

is reached (Figure 1-4). The point of plateau is referred to as the breakpoint and is considered to be the requirement for the test amino acid.

The IAAO method may be an ideal choice to determine amino acid requirements during and following an immune response as it is a rapid technique. The actual experiment can be performed within a 4 hour period, and metabolic adaptation to the diet can be achieved in broilers by providing the test diet 2 days prior to the experiment (Leslie, 2003). Due to the rapidity with which the procedure can be carried out, requirements for specific phases of an innate immune response, such as recovery from the acute phase response, can be characterized. An additional benefit of the IAAO method is that relatively few birds are required. Rather than each test diet being fed to a group of birds, individual birds are used to determine the mean requirement for a population. In addition, birds can be reused for analysis to provide requirements for several ages, for example requirements can be determined for 7, 21, and 42 days of age, thus limiting the amount of birds needed for a single experiment.

IAAO was first developed for use in piglets (Kim et al. 1983; Ball and Bayley, 1984) and has since been used in determining amino acid requirements of humans in both healthy (Humayun et al., 2007) and immune-challenged states (Bross et al., 2000; Courtney-Martin et al., 2002). However in the case of humans, a stable isotope [^{13}C] - phenylalanine tracer (Turner et al., 2006) is used in order to prevent the risk of tissue damage, as opposed to the [^{14}C] - phenylalanine often used in animal studies (Ball and Bayley, 1984; Zello et al., 1993). Although IAAO has been used predominantly to determine amino acid requirements in swine (Ball and Bayley, 1984; House et al., 1998; Shoveller et al., 2003; Moehn et al., 2005), a technique for its use in poultry has been

developed (Ewing, et al., 2001; Tabiri et al., 2002a, Tabiri et al., 2002b) and lysine requirements determined (Coleman et al., 2003). Unlike in humans, this technique has not yet been used in animals to determine requirements for specific amino acids during or following immune challenges.

1.12 Immunity, Selection for Growth, and Amino Acid Requirements: A Synthesis

The interplay between nutrition and immunity is apparent when we look at the effect addition or removal of individual nutrients has on immune responses. Amino acids are essential components of growth and skeletal muscle protein turnover, as well as the innate and adaptive immune systems. Additionally, dietary levels of individual amino acids influence disease recovery and resistance in a variety of species. However, the exact requirement for any amino acid during an immune response has yet to be defined (Klasing, 1998). One technique which may be used to identify requirements during specific phases of an innate immune response is the IAAO method, as measurements can be made over very short periods of time. However the IAAO method relies on voluntary feed intake and as the acute phase response causes birds to become anorexic (Beisel, 1977) measuring requirements during this time becomes difficult. While force feeding the birds would allow adequate consumption of dietary lysine, it would also not accurately portray the normal physiological response to an inflammatory challenge. By determining amino acid requirements during recovery from an acute phase response, voluntary feed intake still occurs, allowing the validation of this method as well as insight into this phase of the immune system. Determining how lysine requirements have changed as a result of

selection for growth potential will allow some understanding of how immune responses have changed as a result. In addition, increasing our understanding of protein turnover during this time may help us understand the mechanisms behind any observed changes.

In addition to increasing our overall knowledge of the chicken immune system understanding the effect that selection for growth rate has had on the immune system in poultry will help the industry to make wiser selection choices as it continues to develop fast-growing lines.. As consumer concerns regarding antibiotic use in the industry grow, we will need to develop alternate methods of combating challenges to the immune system to reduce potential production losses. By determining if a novel method, IAAO, can be used to determine amino acid requirements during recovery from an immune challenge, we will take the first step in establishing amino acid requirements during an immune challenge. As our knowledge regarding these requirements grows, we may be able to develop nutritional programs that help to maintain both immunity and growth as birds are acutely or continuously subjected to challenges from their environment.

The objectives of the following studies were to determine 1) the time course of recovery from LPS injection in 2 week old broiler chicks 2) the lysine requirements during recovery from an acute phase response for 2-week old random-bred and commercial chicks 3) the suitability of IAAO to measure amino acid requirements during brief physiological states 4) protein kinetics, including breakdown, synthesis and flux, and amino acid concentrations during recovery from an acute phase response in the plasma of 2 week old random-bred and commercial chicks.

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Table 1-1: Twenty Standard Amino Acids and Their Nutritional and Chemical Classifications

Amino Acid	Abbreviation	Side Chain pH	Nutritional Status in Poultry
Alanine	ala	Neutral	Dispensable
Arginine	arg	Basic	Indispensable
Asparagine	asa	Neutral	Dispensable
Aspartic Acid	asp	Acidic	Dispensable
Cysteine	cys	Neutral	Conditionally Dispensable
Glutamic Acid	glu	Acidic	Dispensable
Glutamine	gln	Neutral	Dispensable
Glycine	gly	Neutral	Conditionally Dispensable
Histidine	hys	Basic	Conditionally Dispensable
Isoleucine	ile	Neutral	Indispensable
Leucine	leu	Neutral	Indispensable
Lysine	lys	Basic	Indispensable
Methionine	met	Neutral	Indispensable
Phenyalanine	phe	Neutral	Indispensable
Proline	pro	Neutral	Dispensable
Serine	ser	Neutral	Conditionally Dispensable
Threonine	thr	Neutral	Indispensable
Tryptophan	trp	Neutral	Indispensable
Tyrosine	tyr	Neutral	Conditionally Dispensable
Valine	val	Neutral	Indispensable

Adapted from Leeson and Summers, 2001

Table 1-2: Ideal Protein Ratio for the 3 Week Old Chick and 20-40 d Old Broiler

Amino Acid	% of Lysine Requirement	
	Chick[1]	20 to 40 d old broilers[2]
Lysine	100	100
Sulfur Amino Acids	72	75
Threonine	67	63
Valine	77	81
Arginine	105	112
Histidine	31	-
Isoleucine	67	71
Leucine	100	-
Phe + Tyr	105	-
Tryptophan	16	19

Adapted from [1]Firman and Boling, 1998 and [2]Mack et al. 1999

R

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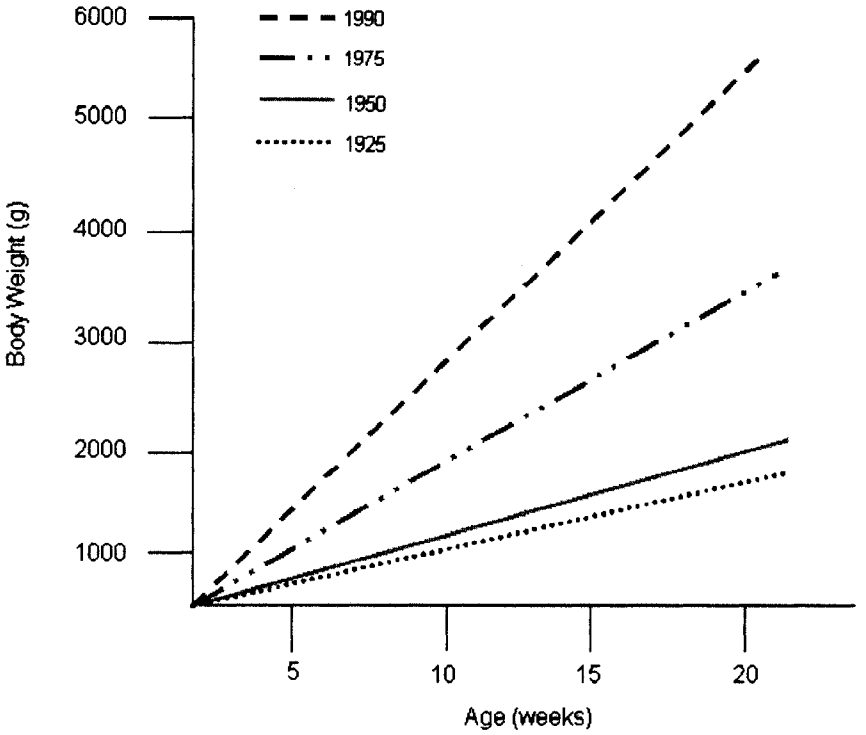
NH2 – CH – COOH

Figure 1-1: Basic Structure of Amino Acids



Adapted from Arthur and Albers in Poultry Genetics, Breeding and Biotechnology

Figure 1-2: Approximate Growth of Broilers From 1990, 1975, 1950 and 1925

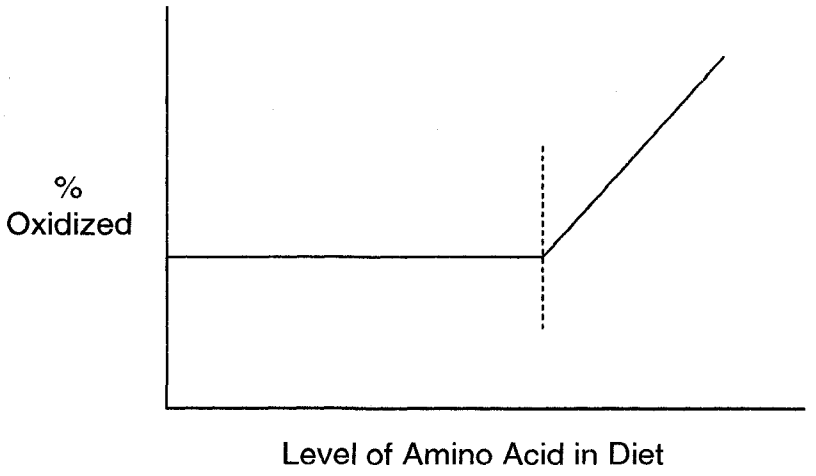


Figure 1-3: Typical Response of Labelled Carbon with Increasing Levels of the Test Amino Acid in the Diet when Performing a Direct Amino Acid Oxidation.
The broken line indicates the breakpoint or requirement for the amino acid.

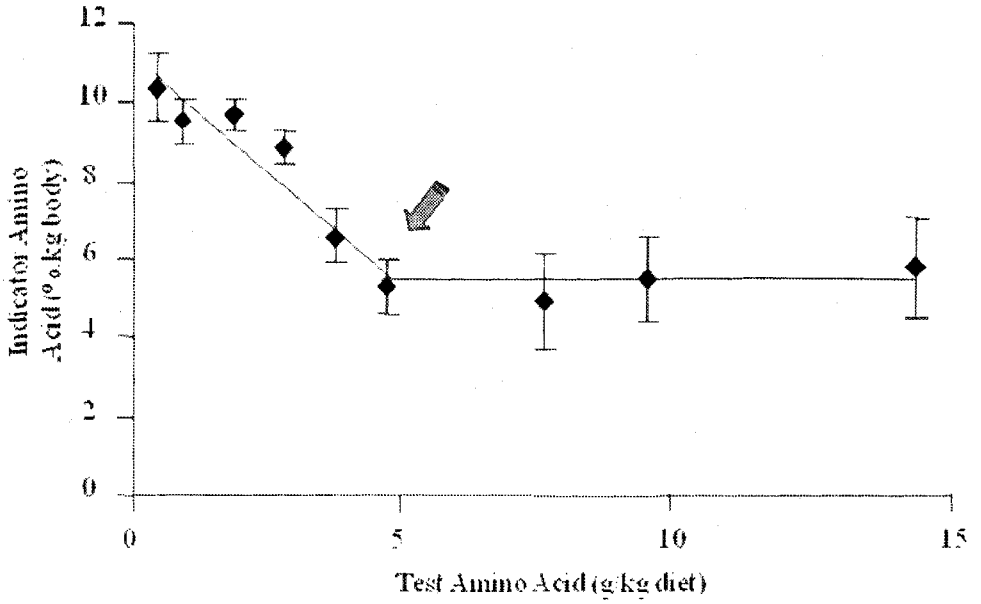


Figure 1-4: Typical Response of Labelled Carbon from the Indicator Amino Acid with Increasing Levels of the Test Amino Acid in the Diet seen when Performing an Indicator Amino Acid Oxidation. The arrow represents the breakpoint or requirement for the amino acid.

2. Determination of Amino Acid Requirements in Two Strains of Broilers during Recovery from an Acute Phase Response

2.1 Introduction

Selection for increased growth rate has allowed the broiler industry to decrease the length of time it takes for modern broilers to reach a target body weight. Although this selection has made raising broilers more efficient, it has also come with consequences. Increased growth rate has been linked with a variety of adverse effects including skeletal abnormalities (Havenstein et al., 1994), reproductive problems (McGary et al., 2003) and an increase in mortality rate due to metabolic diseases such as ascites (Julian, 2000). Although many of the adverse effects have been extensively studied due to their direct effect on production, less attention has been given to the effect selection for growth has had on the immune system.

The immune system can be divided into two main components, the innate system and the adaptive system. The adaptive system provides specific protection to the bird by adapting to and retaining information on invading antigens, however it takes up to seven days post-hatch for this system to mature in broiler chicks (Mast and Goddeeris, 1999).The innate immune system plays an important part in the defence of the bird as it is present from hatch and offers general protection from a variety of pathogens by responding to several carbohydrates common to those pathogens. In addition, the innate immune system helps to guide the adaptive system in the recognition of infectious agents versus non-infectious agents and self (Fearon and Locksley, 1996). In broiler production, birds are exposed to a variety of antigens from a range of environmental sources including, the air, the litter and the water (Apajalahti et al., 2004). The protection offered by the innate immune system during the first seven days post-hatch gives the adaptive

system time to mature, allowing the adaptive system to offer specific protection throughout the bird's life (Wells et al. 1998). Therefore, any unfavourable changes to the immune system resulting from selection for growth have the potential to drastically affect production throughout the bird's lifetime. One method in which the innate immune system manifests itself following trauma, injury, or infection is through the activation of the acute phase response (Suffredini et al., 1999). The acute phase response consists of a series of inflammatory reactions that result in a range of defense mechanisms including fever, anorexia, increased passage of white blood cells to infected tissues, and vasodilation (Suffredini et al., 1999; Baumann and Gauldie, 1994). The acute phase response itself usually happens over 12 to 48 hours (Baumann and Gauldie, 1994) depending on species. Recovery from the acute phase response is characterised by a decrease in body temperature (Dinarello, 1996), increased feed intake and body weight (Fitz-coy and Edgar, 1992), and changing plasma levels of acute phase proteins, such as C reactive protein (Dinarello, 1996) relative to the acute phase response itself.

Work examining the effect of selection for growth on the immune system in poultry is sparse. However, Cheema et al. (2003) compared a 1957 Athens-Canadian random-bred strain and a 2001 commercial strain of broiler chickens and found an increased adaptive response in the random-bred strain whereas the commercial strain displayed an increased innate response. In turkeys, similar work showed no difference in either innate or adaptive immune response between a 1966 random-bred strain and a 2003 commercial strain (Cheema et al. 2007). In our lab, previous work has suggested that 1957- and 1977- random-bred strains had greater total *in vitro* bactericidal activity than a modern commercial Ross 308 strain (Saunders-Blades et al., 2006 unpublished

data). In addition, the 1957 strain showed greater heterophil oxidative burst response than either of the other strains whereas phagocytic uptake of *E. coli* per cell was not affected by strain. As the use of antibiotic growth promoters in the feed often work to limit the amount of bacteria the bird comes into contact with, a decrease in the innate immunity of modern broilers may not pose an immediate threat. However, as consumer concerns grow over the presence of antibiotics in all animal production, the poultry industry may find itself decreasing or eliminating their usage in the near future. This has the potential to hinder production not only in the form of bird mortality but by lowering growth rate through appearance of clinical and sub-clinical infections, causing a constant redirection of resources away from growth. As other aspects of the immune response differ between birds selected for growth and those that remain unselected, it may be fair to assume that their nutritional requirements also differ during this time. By determining requirements of nutrients such as amino acids, we may be able to find ways to implement there supplementation as an alternative to the use of antibiotic growth promoters.

Although amino acid requirements for growth have been extensively studied in broilers (Gous, 1998; Leclercq, 1998; Oviedo-Rondon, and Waldroup, 2002; Labadan et al.,2001;Sklan and Noy, 2003), requirements for the majority of amino acids during an immune response have yet to be defined (Klasing, 1998). The only work that has been done looking at amino acid requirements has been during an acute phase response itself and even that research is limited. Klasing and Barnes (1988) showed that lysine requirements of broiler chickens were lower during an inflammatory response than that required for growth. In broilers, Thronton et al. (2006) and Hale et al. (2004) found that measurements of innate immunity did not differ at different levels of valine and

isoleucine. The lack of research in this area may be related to the long duration usually required to measure amino acid requirements using traditional methods, ranging from 24 hrs to several weeks in length (Borgonha et al. 2002; Kidd et al. 2004; Samadi and Liebert 2007). One practical method of determining amino acid requirements over a short period of time (Ewing et al., 2001), such as recovery from the acute phase response, may be the Indicator Amino Acid Oxidation technique (IAAO).

As consumer concerns regarding the use of antibiotics grow, this makes the recovery phase a likely target for applying alternate measures that could help encourage growth in immune challenged birds thereby reducing the effects on production. Determining how amino acid requirements change during recovery from the acute phase response this will allow us to understand and provide for the nutritional needs of birds at this time.

2.1.1 Project Objectives

The objectives of these experiments were to determine: 1) the time course of the acute phase response in broiler chickens using body temperature and body weight; 2) the suitability of IAAO to measure amino acid requirements during recovery from an acute phase response; 3) if recovery from an acute phase inflammatory response affected dietary lysine requirements and; 4) if genetic selection for growth potential has affected this requirement.

2.1.2 Hypotheses

As lysine requirements were measured during recovery from an immune response, it was hypothesized that the birds challenged with LPS would have a higher lysine requirement as compensatory growth may be occurring as a result of the earlier anorexia induced by

the injection. In addition, the birds not selected for growth were hypothesized to have a lower lysine requirement than those selected for growth as they would be smaller at a particular age than the birds selected for growth, and growing more slowly. National Research Council requirements for lysine have shown to change with selection for growth, increasing from 9g/kg in 1954 to 11g/kg in 1994.

2.2 Materials and Methods

2.2.1 Experiment 1- Acute Phase Response Time Course Study

The purpose of this experiment was to determine the time course of the acute phase response following a pro-inflammatory challenge in broiler chicks using body temperature as an indicator. All procedures were approved by an institutional animal care and use committee. Commercial Ross 308 birds (C) were obtained from Lilydale Hatchery (Edmonton, AB) whereas random-bred (since 1957) chicks (R) were hatched at the Alberta Hatching Egg Producers Hatchery on the Edmonton Research Station (n=48/strain). All birds were wing-banded and individually weighed on the day of hatch and raised in floor pens at the Metabolic Unit on the Edmonton Research Station. Birds were fed a crumbled, nutritionally complete commercial starter diet throughout the experiment. At 10 days of age, birds were individually housed in modified laying hen cages in order to monitor individual bird status (37 R and 39 C).

2.2.1.1 Modified Laying hen cages

Individual cages for Experiment 1 were constructed from metal single bird laying hen cages (18 back to back cages) (Figure 2-1a, b). The tops of the cages on each side were cut open so that they lifted up vertically in order to access the chicks. The floors

and sides of the cages were covered with plastic mesh held in place by zip ties in order to prevent the chicks from escaping. Large metal trays filled with wood shavings were placed side by side underneath the cages in order to catch fecal material. Plastic food and water containers were placed inside the cages and held in place by a fence of plastic meshing in order to stop spillage.

2.2.1.2 Experimental Procedure

At 13 days of age, bird weight and initial rectal temperature were taken immediately prior to the experiment. Approximately half of the birds from each strain (n = 19/strain) were injected intra-abdominally with *Salmonella typhimurium* lipopolysaccharide (LPS; I) whereas the remainder of the birds (n=18 R and 20 C) served as non-injected controls (U). Lipopolysaccharide was prepared by dissolving 100 µg/ml of LPS into 0.9% sterile saline. All LPS solutions were prepared on the day of the injections and all birds in the LPS treatment group were injected with 2 ml of the solution. Each bird was individually weighed and rectal temperature was monitored using a digital thermometer at hourly intervals from the start of the experiment until the injected birds had recovered. Recovery was determined by a return to starting body temperature.

2.2.2 Experiment 2- Determination of Lysine Requirements by IAAO

All procedures were approved by an institutional animal care and use committee. C birds were obtained from Lilydale Hatchery (Edmonton, AB) whereas the R birds were hatched at the Alberta Hatching Egg Producers Hatchery on the Edmonton Research Station. Groups of birds (n=12 R and 12 C per week for 8 weeks) were raised in floor pens with ad libitum access to a nutritionally-complete starter feed. All birds were wing-

banded and weighed on day of hatch and raised in floor pens at the Metabolic Unit at the Edmonton Research Station. At 10 or 11 days of age, birds were placed in individual metabolic chambers and adapted to one of seven semi-purified experimental diets as metabolic adaptation to the test diet takes a minimum of 2 days (Leslie, 2003).

2.2.2.1 Diets

From hatch until 10 days of age, birds were fed a crumbled commercial-type starter ration. From 10 to 14 days of age, birds were given ad libitum access to a semi-purified diet consisting of either 6.07, 7.18, 8.38, 9.38, 10.49,12.14 or 13.24 g/kg of dietary Lys (55% to 120% of NRC (1994) levels for birds of this age; Table 2-1). All amino acids except lysine, methionine and tryptophan were obtained from Sigma Chemicals. Lysine, methionine and tryptophan were provided as feed-grade ingredients. The finished semi-purified diet was extruded at a graded temperature of 60 (inlet) to 70 (outlet)°C, crumbled by hand and stored at -20°C until immediately prior to use to prevent mold. Feed samples were analysed for amino acid composition using high performance liquid chromotography (HPLC) as described previously (Sedgwick et al. 1991).

Prior to each oxidation study, 4 birds per strain, closest to the average body weight and consuming feed, were chosen (a total of 8 birds per oxidation study). Two birds per strain were adapted for three days to each of two diets chosen at random (Figure 2-2). Following the adaptation period, 12 hours prior to each oxidation study (as determined in Experiment 1), one bird per strain x diet treatment was injected with LPS to induce an inflammatory response (2 ml of 100 μg/ml *S. typhimurium LPS*), while the

other served as a non-injected control. Each week, two oxidation studies were performed, at either 13 or 14 days of age. This process was repeated weekly until a minimum of four replicates of each strain x LPS treatment x diet group were obtained. A detailed explanation of the oxidation experiments can be found below.

2.2.2.2 Oxidation Chambers

Oxidation chambers had a total volume of 22.5 L and were constructed of 0.4 cm plexiglass (30 cm deep x 30 cm wide x 25 cm high) (Figure 2-1c, d) allowing the birds to stand and move comfortably inside. Weather stripping (0.6 cm thick) lined the top edges of the chambers to keep lids in place on the chambers during the oxidation. Plastic containers were placed inside the box for feed and water. Removable flooring was constructed of plastic mesh and placed over wood shavings to absorb fecal material. A vacuum pump (Model 1028-1010-G608X, Gast Pump Manufacturing Corp., Edmonton, AB, Canada) drew air through the CO_2 collection system via a small hole in the front of the chamber using Tygon tubing. Flow rate through the chambers was kept at approximately 20L/min.

2.2.2.3 Oxidations

Beginning at 12 hours following LPS injection, an indicator amino acid oxidation study was performed to determine lysine requirements for the two strains of birds. Three gas washing bottles per oxidation chamber (two 500 ml bottles (A) and one 250 ml bottle (B)) were connected in series and filled with 150 ml and 100 ml respectively of CO_2 absorber (monoethanolamine:2-methoxyethanol 1:2 vol/vol). The A bottles were in place to collect the majority of the labeled carbon while the B bottles collected the remainder. All B bottles were weighed prior to the experiment.

Birds were prepared by removing them from the chamber and gavaging a priming dose of L-[1-^{14}C] phenylalanine solution (3 μCi/kg body weight) into the crop using a 3 ml syringe and Tygon tubing. The priming dose was followed every half hour by repeated 1.75 μCi/kg doses throughout the 4 hr experiment. The syringes containing the isotope were weighed prior to and after administering each oral dose in order to accurately determine the exact amount of isotope given to the birds. CO_2 collection was started 30 minutes after the priming dose was administered. Air was drawn from the chamber and passed through the three serially-connected gas washing bottles containing CO_2 absorber. Every 30 minutes, (immediately after each half-hourly dose was given) the bottles were exchanged with a new set of bottles containing fresh absorber. At the end of each 30-minute collection period, the absorber from the two A bottles, per bird,was combined, weighed, and approximately 6 ml were transferred to a 7 ml scintillation vial. The B bottle for each bird was weighed and an aliquot of its contents transferred to an additional 7 ml scintillation vial. A 1 ml aliquot of each 7 ml sample collected was pipetted to a new 7 ml scintillation vial, weighed, and 2 ml (liquid scintillation cocktail (Atomlight, Dupont Canada, Mississauga, ON, Canada) was added. Radioactive decay per minute (DPM) of the samples was then counted using a liquid scintillation counter (Beckman Instruments Inc, Irvine, CA, USA) in order to allow calculation of % dose oxidized. At each level of dietary lysine, there were four replicates for each strain-LPS treatment. Individual bird and feed weights were taken before LPS injection and before and after each 4-hr oxidation period

2.2.2.4 Calculations

Infused DPM were calculated for each oral (priming and repeated) doses by:

$$DPM/Kg = \frac{((\text{Initial Syringe Weight} - \text{Final Syringe Weight}) * [\text{Isotope Solution}])}{\text{Bird Weight}}$$

Where:
Initial Syringe Weight = the weight (g) of syringe after adding the isotope solution
Final Syringe Weight = the weight (g) of syringe after giving an oral dose of the isotope solution
[Isotope Solution] = The concentration (DPM/g) of the isotope solution
Bird Weight = the weight (Kg) of the bird after the oxidation

DPM collected (every 30 min) was calculated by:

$$DPM = \frac{(DPM\ A\ * Wt\ A)}{\text{Sample Wt A}} + \frac{((DPM\ B\ ) * (\text{Tare B} - Wt\ B))}{\text{Sample Wt B}}$$

Where:
DPM A = calculated decays per minute of the 1 ml subsample from the A bottles
Sample Wt A = the weight (g) of the subsample from the A bottles
Wt A = the combined weight (g) of the absorber from the two A bottles
DPM B = calculated decays per minute of the subsample from the B bottles
Sample Wt B = the measure weight (g) of the subsample from the B bottles
Wt B = the weight (g) of the absorber from the B bottle
Tare B = the weight (g) of the empty B bottle

Dose Oxidized was calculated by:

$$\frac{\text{DPM Collected (per 30min))}*100}{\text{DPM infused (per 30 min)}} = \%\ \text{Oxidized}$$

2.2.3 Statistical Analysis

2.2.3.1 Experiment #1

The main effects were LPS injection, time and strain, with each bird being the experimental unit. The effect of LPS on body temperature and body weight and the interaction of strain and injection and time were analyzed using the Mixed procedure of SAS with a significance value set at $P \leq 0.05$.

2.2.3.2 Experiment #2

Body weights were analysed using ANOVA in the GLM procedure of SAS (SAS Institute, 1999) with a significance level set at $P \leq 0.05$. The Mixed procedure of SAS was not used as measurements were not repeated within a single bird for this experiment. The main effects were dietary lysine level, LPS injection and strain with each bird being

the experimental unit. Two different methods were used to estimate lysine requirements in the non-linear procedure of SAS (Coleman et al. 2003). A minimum of four birds were used to calculate the percent of phenylalanine oxidized at each dietary level. Using the first program per cent dose phenylalanine oxidized was regressed on dietary lysine content and the breakpoint was adjusted iteratively until a minimal residual mean square was found. The second program simultaneously made measurements of both slope and breakpoint (Goonewardene, personal communication). This was done as in order to estimate variability as multiple measurements were not taken within birds. Treatments were compared using a T-test in the GLM procedure of SAS with a significance level set a $P \leq 0.05$.

2.3 Results

2.3.1 Experiment 1

Strain significantly affected temperature ($P<0.0001$) with the R birds having a higher temperature at all time points after 4 hr post injection than the C birds (Figure 2-3). LPS treatment also significantly affected temperature of the birds ($P < 0.0001$). Injected birds had a higher temperature than un-injected birds. There was a significant interaction of LPS treatment and time, injected birds of both strains started to show signs of fever 4 hr post injection, with temperature declining from approximately 11 to 18 hr post injection. There were no changes in the un-injected birds' temperatures throughout the experiment. All birds were fully recovered as determined by return to normal body temperature by 18 hr post-injection.

2.3.2 Experiment 2

The C strain had higher BW than the R strain throughout the experiment ($P \leq 0.0001$) (Table 2-2). At hatch, the R birds had a significantly lower body weight than the C birds. Before injection on day 12 or 13, the R birds on all diets had a significantly lower body weight than the C birds, this is most likely due to the slower growth rate of the birds. There was also a 2-way interaction of strain and diet, most likely caused by the fact that the C birds had a significantly higher body weight on the 120% diet whereas the R birds body weight did not differ due to dietary treatment. Before the oxidation at 12 hours following LPS injection, there was a three-way interaction of LPS treatment, diet and strain on BW (P=0.0004). CU birds fed the 120% diet had a significantly higher BW than all other birds and birds in the CU treatment group showed a trend of increasing body weight with increasing dietary lysine. In the CI birds this trend did not exist, and CI birds on the 65%, 75% and 110% diets had the highest BW, with birds fed the 75% diet reaching BW similar to the CU birds in several of the dietary treatment groups (Table 2-2). The R birds had similar body weights regardless of diet or LPS treatment. Following the oxidation, a similar interaction was seen with the CU birds fed the 120% diet having a significantly higher BW than all other birds. The increased body weight with increasing dietary lysine was observed in the CU birds, whereas again this pattern was not seen in the CI birds. However following the oxidation, the RI birds fed the 120% and75% diets and RU birds fed the 120% diet had a significantly greater BW then RU birds fed the 95% diet. Looking at the BW data as a % of the pre-injection BW, in order to remove the effect of the faster weight gain in the C birds, the effect of diet on body weight disappears (Table 2-3). basedon % of pre-injection BW, both before and after the oxidation, the CU

birds had a significantly higher increase in BW than the CI birds ($P \leq 0.0001$) whereas the RI birds and the RU birds did not differ ($P = 0.4498$).

The mean Lys requirements for RI and RU birds were 9.98 ± 0.26 and 8.32 ± 0.11 g/kg diet, respectively ($P = 0.0231$; Figure 2-4). Mean lysine requirements for the CI and CU birds were 11.95 ± 1.09 g/kg diet and 10.14 ± 1.14 g/kg diet, respectively ($P = 0.7307$) (Figure 2-4). Across LPS treatment, the C birds had a higher lysine requirement than the R birds ($P \leq 0.0001$).

2.4 Discussion

2.4.1 Experiment 1

The higher temperature of the injected birds was expected as LPS injection has been shown previously to induce fever in broilers (Baert et al. 2005). Based on the results of this experiment, birds in Experiment 2 were injected 12 hr prior to the oxidation to ensure that birds were in the recovery phase during the oxidation studies. An increase in body temperature and a decrease in feed intake have both been associated with an LPS-induced acute phase response in poultry (Xie et al., 2000). Birds injected with LPS had elevated temperatures 3 hr post injection but not at 12 or 24 hr post injection (Xie et al., 2000). A separate study agreed with the results found in the present study with LPS causing a peak in fever at 12 hr post injection (Leshchinsky and Klasing, 2001). Differences in time course between Xie et al. (2000) and the current study could be a result of bird genetics or age, or the biological activity of the LPS used. Birds used by Xie et al. (2000) had an average body weight of 0.69 kg at 3 weeks of age whereas birds

used in the current study were only 2 weeks of age and ranged from 0.144 kg to 0.460 kg, depending on strain.

2.4.2 Experiment 2

The results of this experiment demonstrate that commercial and random-bred birds differed in their recovery from an inflammatory challenge. The higher body weight at hatch of the commercial birds was most likely due to strain differences between birds as larger hatch weights have been found in birds more heavily selected for growth (Wolanski et al., 2006). The strain x diet interaction seen before the injection was most likely due to the significantly higher body weight of the C birds on the 120% diet while the R birds did not differ in body weight due to dietary treatment. The significantly higher body weight of the CU birds on the 120% diet and the trend of increasing body weight with increasing dietary lysine were expected, however it was hypothesized that both the R and C birds would show this trend. Broilers fed incremental increases in dietary lysine up to the requirement have been previously shown to increase body weight (Sterling et al., 2003). The reason this trend was not seen in the CI birds could be related to their lysine needs. This suggests that the commercial injected birds required less lysine than the un-injected birds. However if this were true, we would expect to see an increase followed by a plateau in body weight as dietary lysine levels approach and surpass the requirement, yet there did not seem to be any pattern to the effect of dietary lysine on body weight gain. The highest body weights were seen in the 65%, 75% and 110% treatments. The difference in BW in these groups could also be a result of the differential inflammatory responses and variable individual weight gain during the acute phase response.

However, BW was not affected in the R birds regardless of treatment or diet before the oxidation. The significantly higher weight of the RI birds fed the 120% and 75% diets and RU birds fed the 120% diet compared to the RU birds fed the 95% diet is most likely because 2 birds within that dietary treatment had lower BW than the rest of the birds in the experiment and therefore did not gain as much weight throughout the oxidation. Decreases in weight gain have been previously seen in birds injected with LPS compared to un-injected controls (Xie et al. 2000) however, the differential effect of dietary lysine on BW between R and C birds has yet to be studied.

Looking at body weight as a % of pre-injection body weight both before and after the oxidation removed the effect of diet, suggesting that the birds actually showed similar proportional weight gain within strain and treatment regardless of diet (Table 2-3). The CU birds gained significantly more BW as a percentage of the pre-injection BW, both before and after the oxidation, than any of the other strain x LPS treatment groups (Table 2-3).

The lysine requirements for the CU birds (10.14 g/kg) were higher than for the RU birds (8.32 g/kg) as hypothesized. These values for the CU birds and the RU birds agree with the NRC requirements for 1994 (11g/kg) and 1954 (9g/kg) respectively. Despite the fact that the majority of CI birds on all diets had a lower BW than the CU birds, their lysine requirements were not affected by LPS injection. Although the RI and RU birds body weights were not affected by LPS injection, the RI birds had a higher lysine requirement than the RU birds. The differences seen between the two strains could be due to a differential effect of the inflammatory response on the C and R birds, perhaps suggesting a larger redistribution of nutrients from growth to immunity in the C birds.

The extent to which lysine requirements have been studied in relation to an inflammatory response in broilers is limited. Lysine given to broilers in excess of the dietary requirement for growth did not significantly improve either cellular immunity or antibody response to sheep red blood cells (Kidd et al. 1997). However, when measured by cutaneous basophil hypersensitivity stimulated by phytohemagglutinin a deficiency in lysine affected cell-mediated responses in poultry, such as T-cell function. (Chen et al. 2003). A similar response has been seen in mice with malignant tumors, where limitation of lysine produced a slight depression of both antibody and cytotoxic cellular immunity, but this depression was not as pronounced as those seen in response to other amino acid deficiencies (Jose and Good, 1973). This seems to suggest that lysine is a required nutrient during an immune response, but may only be needed at or below the levels required for maximum growth.

Lipopolysaccharide injection has been previously observed to decrease lysine requirement of broilers during an immune challenge (Klasing and Barnes, 1988), however we observed an increase during the recovery phase in random-bred, but not in modern broiler chickens. It is possible that the higher lysine requirement of the RI birds as compared to the RU was a result of compensatory growth, as this has been observed previously in poultry immediately following recovery from an infection (Newcombe et al., 1992). In humans, it has been found that during the recovery phase of an innate immune response, a larger amount of body protein can be retained as compared to healthy subjects, suggesting an increase in protein synthesis during this time (Kurpad, 2006). This may also be true in poultry, as both Lippens et al. (2002) and Leeson and Zubair (1997) found that compensatory growth improves nitrogen retention following

periods of restricted feed intake. If this increase in retained protein is coupled with an increase in protein synthesis as a result of compensatory growth, this would likely lead to an increased requirement during recovery from the acute phase response. If this were true, it would be expected that the BW of the RI birds would also reflect this, however the RI birds BW did not increase at a greater rate than the RU birds during the oxidation (Table 2-3). The CI birds however gained BW at a much slower rate then the CU birds (Table 2-3) suggesting that compensatory growth was not occurring in these birds.

Differences in time course of the acute phase response between the two strains may also be a possible explanation. Recovery may happen at a different rate between the two strains and as requirements were only measured at a single time point this could help to explain the differences in lysine requirements between the two strains. However, when we look at the time-course data from Experiment 1, there was no strain by time interaction. This suggests that, at least in temperature based recovery, the strains have a similar timeline.

Another explanation could be that the mechanisms controlling recovery from an acute phase response differ between R and C birds. Mechanisms between R and C strains have been shown to differ during an innate immune response. Cheema et al. (2003) showed that commercial birds may have an increased innate response, as measured by phagocytosis of sheep red blood cells by macrophages, compared to random-bred birds (Cheema et al. 2003). Data from our lab suggests that birds not selected for growth since 1957 or 1977 had a greater total *in vitro* bactericidal activity than a modern commercial Ross 308 strain (Saunders-Blades et al., 2006, unpublished data). The 1957 strain also had a greater heterophil oxidative burst response than either of the other strains; however,

phagocytic uptake of *E. coli* by white blood cells *in vitro* was not different among any of the 3 strains. Leshchinsky and Klasing (2001) also found that broilers selected for growth rate showed lower mRNA levels of the cytokines myelomonocytic growth factor, interleukin-1B and Interferon γ compared to layer-type birds not selected for growth, suggesting less capacity to generate an inflammatory response in the broilers. Therefore, it may be safe to assume that mechanisms controlling how the birds recover from an innate immune response also differed between strains, which resulted in a higher lysine requirement in RI birds. Repeated studies with birds of the same age or research looking at differences in protein turnover, or acute phase protein concentration may help to clarify this by examining some of the mechanisms behind these changes. In addition, a small number of birds were used in the majority of these studies (4 birds per strain per treatment), using a larger number of birds may allow for a greater sample of the population and reduce the variance between birds.

A common method used to measure amino acid requirements in both poultry and humans is through growth or nitrogen balance studies (Barkley and Wallis, 2001; see Pencharz and Ball, 2003 for a review; Zello et al., 2003; Samadi and Liebert, 2007). Nitrogen balance and growth are considered the standards for determining amino acid requirements and have been the most widely used in many species. In the nitrogen balance technique, groups of birds are adapted to otherwise nutritionally-complete diets that vary in the level of a test amino acid. After 5 to 10d of adaptation to the test diet, feces are collected for an additional 5 to 10dafter which nitrogen intake and excretion is measured and the difference is equivalent to the nitrogen balance of the animal (Tzeng and Davis, 1980). Although this method is suitable for determining amino acid

requirements during time periods in the animal for which the animal's requirements are relatively stable, the lengthy experimental method makes it difficult to measure requirements which change over the course of a few hours or days. In the 24 hr amino acid balance method, the balance of the test amino acid is measured in response to graded levels of the test amino acid in the diet over 24 hr (Pencharz and Ball, 2003). The 24 hr time period may not be short enough to measure the changing requirements during the acute phase response.

The IAAO method may be an ideal choice to determine requirements following an acute phase immune response, as it can be performed within a 4 hour period (Leslie et al. 2003). An additional benefit of the IAAO method is that relatively few birds are required. A minimum of 4 birds per treatment are necessary to determine requirements (Coleman et al. 2003). However due to the results in this chapter, as well as the subsequent chapter (Chapter 3) demonstrating that recovery from the acute phase response may vary significantly amoung birds, determining requirements during this time may require a larger sample size. Because the acute phase response causes anorexia (Beisel, 1977), and in IAAO the test amino acid is provided in the diet, accurate results depend on voluntary feed consumption by the bird; thus it may be difficult to use IAAO during the acute phase. However, during recovery from the acute phase, the birds will resume eating, and IAAO can be used. The current experiment has shown that IAAO may be useful to determine amino acid requirements during recovery from an acute phase response; this will allow this method to be used to further our knowledge on the avian immune system and changes that have occurred as a result of selection for growth.

2.5 Conclusion

In conclusion, continual selection for growth rate of broiler chickens has led to many unintended consequences in modern broilers. There is only limited research available on how selection has affected the innate immune system and the requirement for amino acids of broilers. While the expected increase in temperature due to an inflammatory challenge was seen in the injected birds in experiment 1 (Baert et al. 2005), there did not seem to be a difference in temperature responses between the two strains. It has previously been shown that lysine requirements during an inflammatory response are lower than what is required for growth (Klasing and Barnes, 1988). However, when we examined requirements during recovery from the acute phase response in the current study, the RI birds had a higher lysine requirement than the RU birds, whereas the commercial birds' requirements were not affected by LPS injection. This could be a possible result of differences in compensatory growth or metabolic responses during the recovery phase between the commercial and random-bred birds. Existing research on protein metabolism during recovery from the acute phase response is limited; hence, further investigation needs to be done before this can be confirmed. In addition the usefulness of the IAAO technique for determining amino acid requirements over very short metabolic periods, such as recovery from the acute phase response, was verified, allowing the future use of this technique to help us expand our knowledge on the broiler immune system. Although strain and LPS injection have both affected the lysine requirement of the bird, we do not yet know the mechanisms behind these changes.

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Table 2-1: Composition of the Semi-purified Diets for Experiment 2

Ingredients	Dietary Lysine (g/kg diet)						
	55%	65%	75%	85%	95%	110%	120%[5]
	g/kg diet						
Corn Gluten Meal	100	100	100	100	100	100	100
Soybean Meal	190	190	190	190	190	190	190
Cornstarch	400	400	400	400	400	400	400
Canola Oil	40	40	40	40	40	40	40
Sucrose	100	100	100	100	100	100	100
Cellulose	40	40	40	40	40	40	40
Vitamin - Mineral Premix[1]	5	5	5	5	5	5	5
Choline Chloride Premix [2]	5	5	5	5	5	5	5
Limestone	19	19	19	19	19	19	19
Dicalcium Phosphate	20	20	20	20	20	20	20
Total AA[3]	81	81	81	81	81	81	81
Analyzed Nutrient Composition							
	Kcal/Kg or %						
Metabolizable Energy[4]	3,207	3,202	3,197	3,192	3,187	3,179	3,174
Calcium[4]	0.90	0.90	0.90	0.90	0.90	0.90	0.90
Available Phosphorus[4]	0.45	0.45	0.45	0.45	0.45	0.45	0.45
Crude Protein	21.78	21.78	21.78	21.78	21.78	21.78	21.78
Methionine	0.52	0.51	0.49	0.47	0.50	0.52	0.48
Methionine + Cysteine	0.94	1.04	0.92	0.96	0.92	0.93	0.90
Lysine	0.56	0.61	0.79	0.92	1.03	1.23	1.33
Threonine	0.91	0.95	0.86	0.94	0.84	0.83	0.96
Arginine	1.48	1.50	1.37	1.38	1.53	1.39	1.43
Glysine + Serine	1.13	1.24	1.29	1.15	1.19	1.13	1.29
Serine	0.72	0.78	0.76	0.69	0.68	0.71	0.72
Histidine	0.36	0.41	0.42	0.38	0.37	0.41	0.38
Isoleucine	0.92	0.87	0.94	0.91	0.94	0.88	0.90
Phenyalanine	0.87	0.82	0.89	0.84	0.91	0.90	0.85
Valine	1.00	0.99	1.08	1.08	1.09	1.10	1.04

[1] The Vitamin - Mineral Premix provided the following per kg of diet: Vitamin A, 10,000 IU; Vitamin D_3, 2,500 IU; Vitamin K, 2.0 mg; Pantothenic Acid, 14 mg; Riboflavin, 5.0 mg; Folacin, 0.80 mg; Biotin, 1.8 mg; Niacin, 65 mg; Thiamine, 2.0 mg; Pyridoxine, 4.0 mg; Vitamin B_{12}, 0.015 mg; Vitamin E, 35 IU; Mn, 70 mg; Cu, 8.5 mg; Zn, 80 mg; Se, 0.10 mg; Fe, 100 mg; Choline, 1000 mg.

[2]Choline premix provided choline at 100 mg/kg diet

[3] Total AA included Asp 240 g; Tryp, 5.3 g; Thr, 19.2g; Met, 26.4g; Lys and glut were included at different amounts in each diet to allow it to be isonitrogenous and contain the appropriate amount of lys. Lys and glu included at 0 g and 43.7 g respectively for the 55% diet; 6.7 g and 37g for the 65% diet; 13.4 g and 30.3g for the 75% diet; 20.2 g and 23.5 g for the 85% diet; 26.9 g and 16.8 g for the 95% diet; 37 g and 6.7 g for the 110% diet; 43.7 g and 0 g for the 120% diet.

[4]Calculated values.

5 Represents 6.07, 7.18, 8.38, 9.38, 10.49,12.14 or 13.24 g/kg of dietary Lys (55% to 120% of NRC (1994) levels for birds of this age

Table 2-2: The Effect of Diet, LPS injection, and Strain on Body Weights of Random-bred and Commercial Birds[1]

Strain[2]	LPS[3]	Diet[4]	Hatch	Before[5] injection	Before[6] Oxidation	After[7] Oxidation
			----------BW (g)----------			
C	U	55	40.2^{bc}	247.8^{a}	279.7^{cdef}	287.0^{def}
		65	44.3^{a}	271.3^{a}	285.2^{cd}	298.0^{cd}
		75	44.8^{ab}	266.8^{a}	305.2^{bc}	313.0^{bcd}
		85	45.4^{a}	272.4^{a}	305.4^{bc}	323.2^{bc}
		95	47.2^{a}	271.9^{a}	311.7^{b}	326.2^{b}
		110	43.5^{ab}	270.2^{a}	317.6^{b}	322.6^{bc}
		120	44.8^{ab}	313.1^{b}	354.3^{a}	363.7^{a}
C	I	55	40.2^{a}	254.3^{a}	256.4^{f}	265.9^{f}
		65	49.1^{a}	265.1^{a}	284.6^{cd}	295.9^{d}
		75	41.3^{ab}	273.7^{a}	294.2^{bc}	309.2^{bcd}
		85	49.7^{a}	273.7^{a}	250.5^{f}	270.8^{ef}
		95	46.7^{a}	228.2^{a}	260.9^{ef}	274.7^{ef}
		110	44.2^{ab}	265.5^{a}	282.0^{cde}	290.0^{de}
		120	43.5^{ab}	255.9^{a}	262.5^{def}	268.2^{ef}
R	U	55	36.7^{cd}	113.2^{b}	115.8^{g}	118.7^{gh}
		65	34.7^{cd}	111.9^{b}	124.0^{g}	126.4^{gh}
		75	40.1^{cd}	115.1^{b}	124.5^{g}	126.1^{gh}
		85	36.3^{cd}	116.1^{b}	111.2^{g}	116.5^{gh}
		95	36.6^{cd}	109.3^{b}	105.0^{g}	105.8^{h}
		110	36.3^{d}	105.8^{b}	123.6^{g}	126.1^{gh}
		120	36.3^{cd}	119.6^{b}	129.3^{g}	135.4^{g}
R	I	55	37.0^{cd}	112.2^{b}	113.6^{g}	118.2^{gh}
		65	40.0^{cd}	97.1^{b}	110.6^{g}	114.2^{gh}
		75	35.5^{cd}	115.9^{b}	124.5^{g}	126.9^{g}
		85	36.6^{cd}	102.6^{b}	109.8^{g}	112.9^{gh}
		95	37.4^{cd}	116.7^{b}	118.6^{g}	122.3^{gh}
		110	36.6^{d}	102.5^{b}	107.4^{g}	112.2^{gh}
		120	36.3^{cd}	116.4^{b}	126.7^{g}	132.0^{g}
Pooled SEM			0.58	7.24	7.15	7.45
Treatment			<0.0001	<0.0001	<0.0001	<0.0001
Diet			<0.0001	<0.0001	<0.0001	<0.0001
Strain			<0.0001	<0.0001	<0.0001	<0.0001
Diet*Treatment			0.5644	0.9752	<0.0001	<0.0001
Diet*Strain				<0.0001	<0.0001	<0.0001
LPS Treatment*Diet*Strain			0.9857	0.1949	0.0004	0.0002

[a-h] Means within a column with common superscripts are not different (P>0.05)

[1] Means represent a minimum of 4 birds per strain by LPS by diet interaction

[2] C=commercial strain; R = random-bred strain (not selected for growth since 1957)

[3] LPS = Lipopolysaccharide; I = birds injected intra-abdominally at 11 or 12 d of age with 2 ml of a 100 μg/ml solution of *S. typhimurium* LPS; U = birds not injected with LPS

[4] Represents 6.07, 7.18, 8.38, 9.38, 10.49,12.14 or 13.24 g/kg of dietary Lys (55% to 120% of NRC (1994) levels for birds of this age

[5] Injections occurred at 11 or 12 d of age, 12 hours prior to the oxidation studies at 12 or 13 d of age, body weights were taken immediately prior to injection

[6] Oxidations occurred 12 hours following injection, body weights were taken immediately prior to the oxidation

[7] Oxidations lasted 4 hours and 30 minutes; birds were given free access to feed and water throughout the oxidation, body weight was measured immediately following the oxidation.

Table 2-3: The Effect of LPS Injection and Strain on Body Weights of Random-bred and Commercial Birds on a % of Before Injection Weight

Strain	LPS Treatment	Weight Before Injection (g)	Weight Before Oxidation (as a % increase of Before Injection Weight)	Weight After Oxidation (as a % increase of Before Injection Weight)
C	I	259[a]	5.00[b]	9.44[b]
C	U	268[a]	15.88[a]	19.98[a]
R	I	108[b]	7.58[b]	10.78[b]
R	U	113[b]	7.63[b]	10.51[b]
Pooled SEM		7.24	1.240	1.243
P Value[1]				
Strain		<0.00001	0.2768	0.1180
Treatment		0.1276	0.0381	0.0496
Strain*Treatment		0.6504	0.0401	0.0390

[1] There were no significant differences for diet or any of its interactions.
[a-c] Means within a column with common superscripts are not different (P>0.05).
[2] Means represent a minimum of 4 birds per strain by LPS by diet interaction.
[3] C=commercial strain; R = random-bred strain (not selected for growth since 1957).
[4] LPS = Lipopolysaccharide; I = birds injected intra-abdominally at 11 or 12 d of age with 2 ml of a 100 μg/ml solution of *S. typhimurium* LPS; U = birds not injected with LPS.
[5] Dietary level of lysine, corresponding to 55% to 120% of NRC (1994) recommended levels of dietary lysine for broiler chickens.
[6] Injections occurred at 11 or 12 d of age, 12 hours prior to the oxidation studies at 12 or 13 d of age, body weights were taken immediately prior to injection.
[7] Oxidations occurred 12 hours following injection, body weights were taken immediately prior to the oxidation.
[8] Oxidations lasted 4 hours and 30 minutes birds were given free access to feed and water throughout the oxidation, body weight was taken immediately following the oxidation.

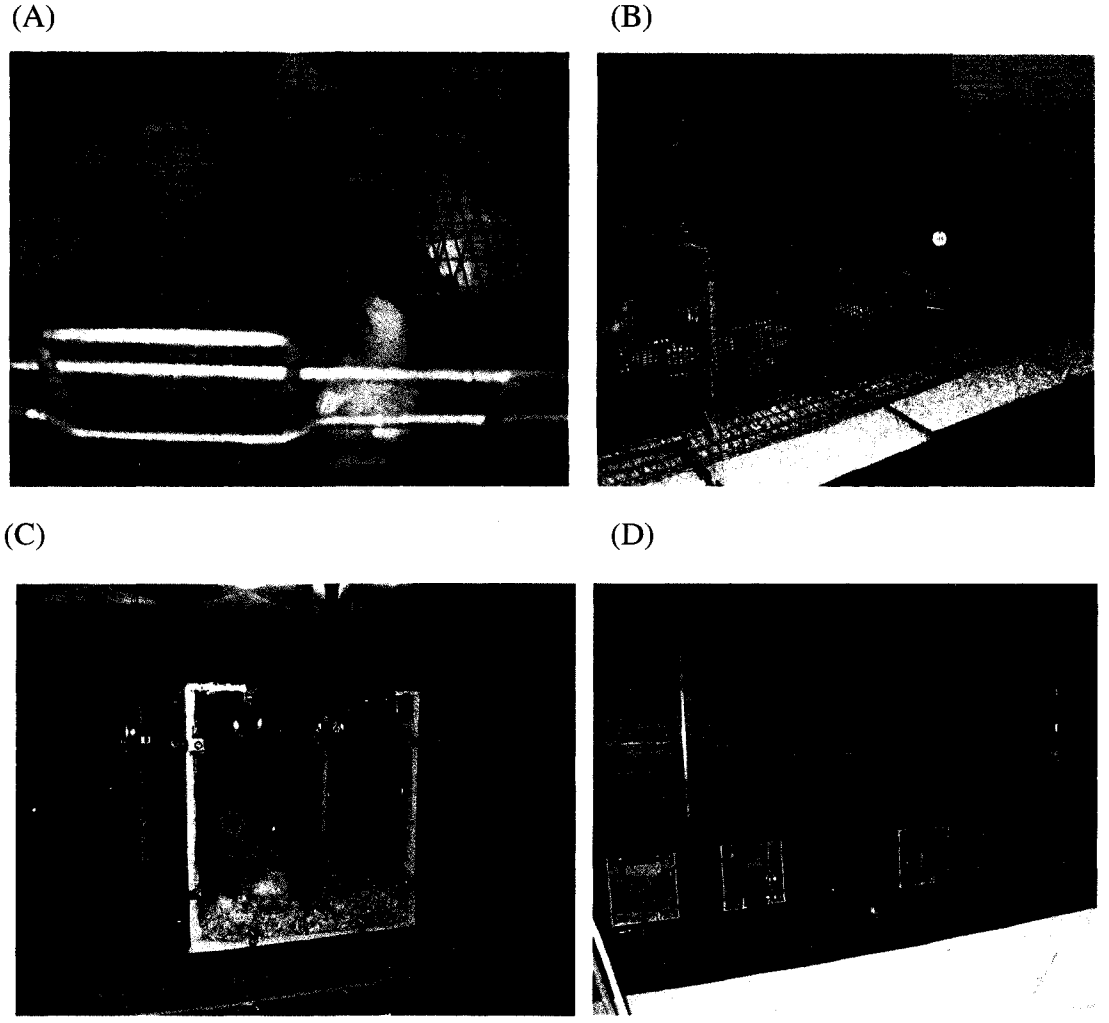

Figure 2-1: (A) top view of a single pen in the modified laying hen cages housing a 13 day old broiler (B) Lateral view of modified laying hen cages (C) Individual oxidation chamber housing a 13 day old broiler chick (D) Lateral view of oxidation chambers.

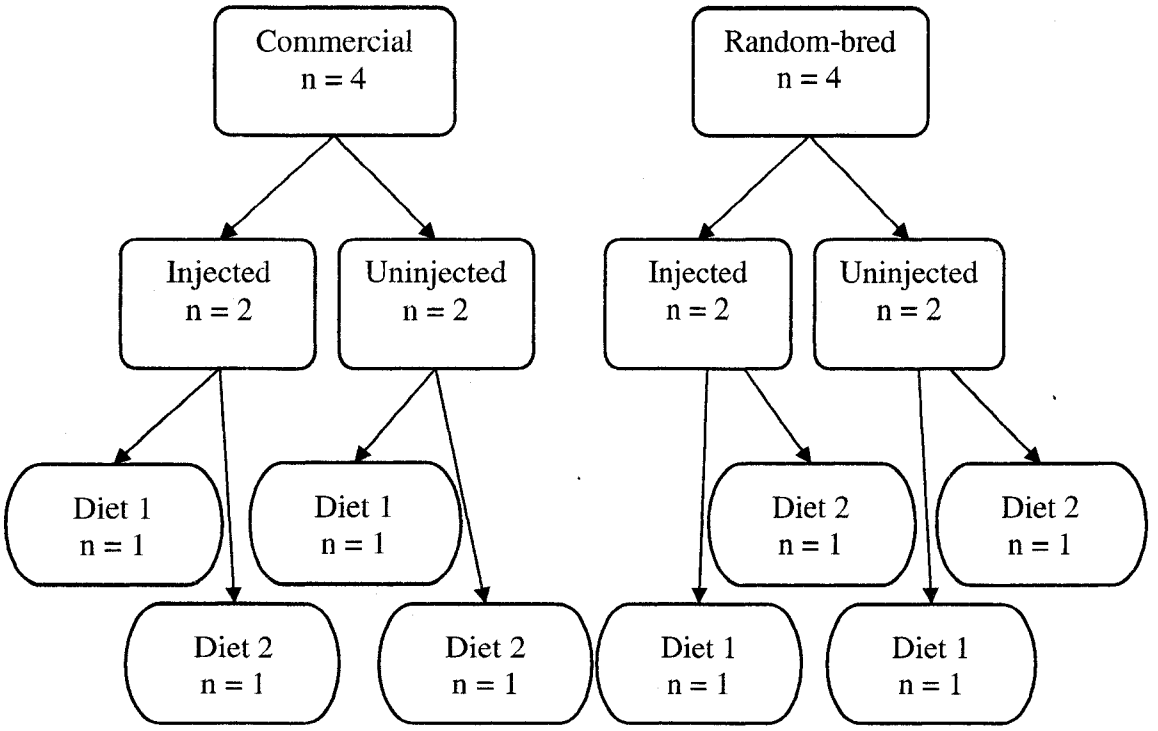

Figure 2-2: Flow Chart Showing the Strain x Treatment x Diet Grouping of Broiler Chicks During each Oxidation. Diet 1, Diet 2 represent two random diets fed at every oxidation containing 6.07, 7.18, 8.38, 9.38, 10.49,12.14 or 13.24 g/kg of dietary lysine (55% to 120% of NRC (1994) levels for birds of this age). Injected birds were injected with 2ml of100µl/ml *S. typhimurium* lipopolysaccharide. This scheme was repeated with randomly-assigned diets until a minimum of 4 replicates per strain x diet x injection treatment was obtained.

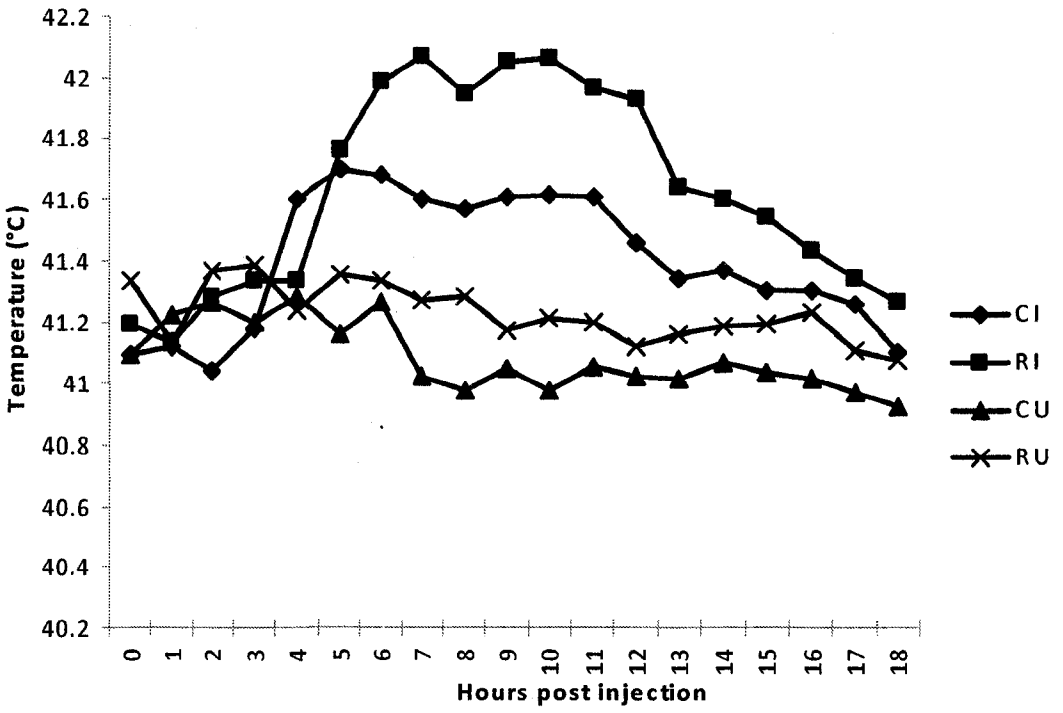

Figure 2-3: Effect of an Inflammatory Response on Body Temperature of Commercial and Random-bred Broilers in Experiment 1. C = commercial strain; R = random-bred strain (not selected for growth since 1957); I = birds injected intra-abdominally with 2 ml of a 100 µg/ml solution of lipopolysaccharide; U = birds not injected with *S. typhimurium* LPS; LPS = Lipopolysaccharide.

[1] P values: Strain – P<0.0001; LPS Treatment - P<0.00001; Strain * Treatment – P=0.1081; Time – P<0.00001; Strain*Time – P=0.2740; LPS Treatment*Time - <0.00001; Strain*LPS Treatment*Time – P=0.8352.

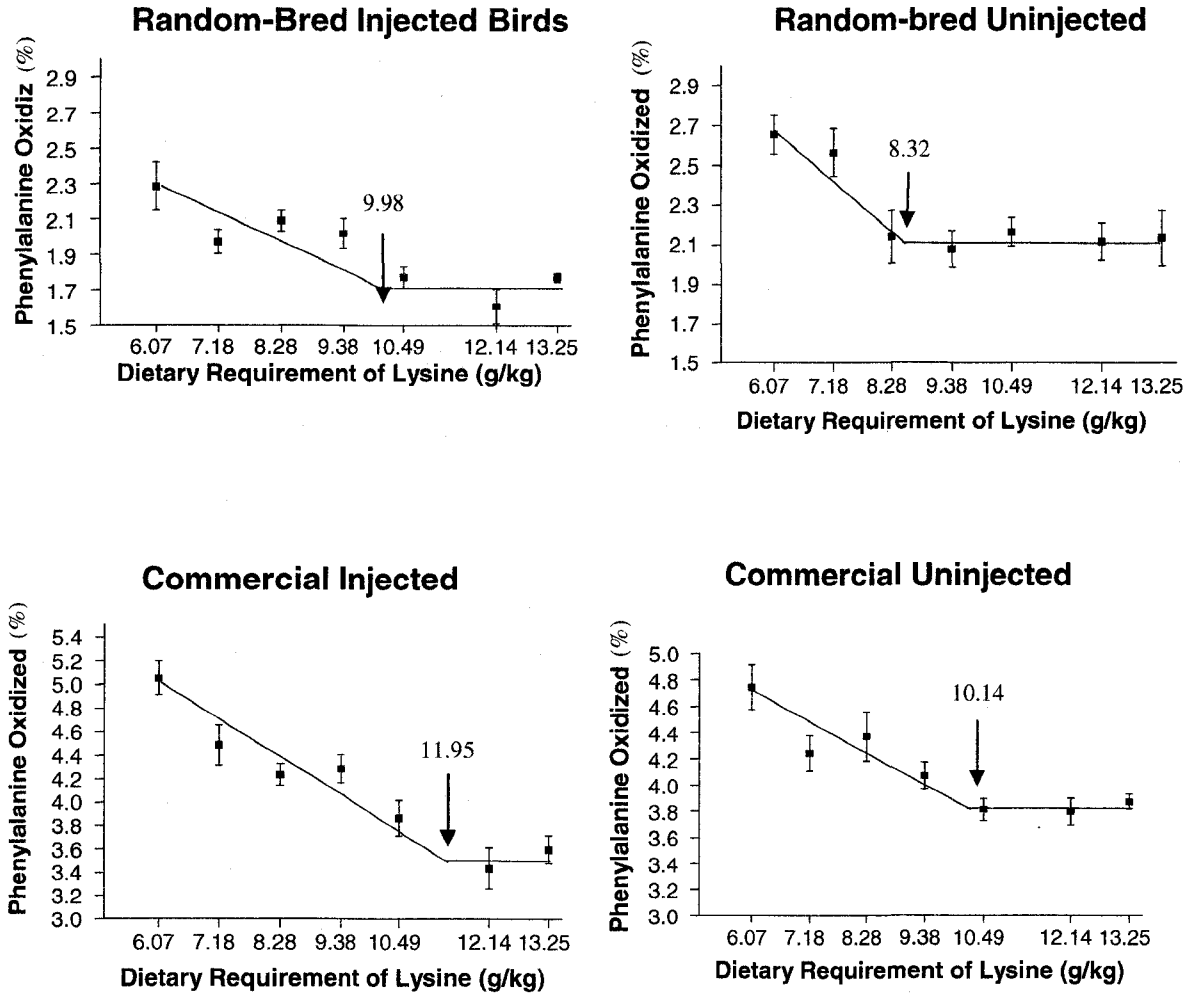

Figure 2-4: Lysine Requirements as Determined by IAAO of 2-week Old Random–bred and Commercial Broilers. Injected birds were injected intra-abdominally at 11 or 12 d of age with 2 ml of a 100 μg/ml solution of *S. typhimurium* lipoplysaccharide. Oxidations occurred 12 hours following injection, body weights were taken immediately prior to the oxidation. Arrows represent the lysine requirement in g/kg diet.

3 Effect of Strain and Immune Status on Whole Body Protein Kinetics, Plasma Amino Acid Profile and Dietary Lysine Requirements in Broilers as Determined by Indicator Amino Acid Oxidation

3.1 Introduction

Commercial broiler chickens have been continuously selected for increased growth rate over the last several decades (Emmerson, 1997). Although this has resulted in both increased rates of body weight gain and feed efficiency of the birds, it has also caused several undesirable effects on production. Selection for growth has been associated with several metabolic (Julian, 2000), skeletal (Havenstein et al., 1994) and reproductive problems (McGary et al., 2003). Selection for growth rate has also been associated with changes in immunity (Cheema et al. 2003), however considerably less is known about its effects on the immune system. The acute phase response is one manifestation of the innate immune system and consists of a series of inflammatory reactions that result in a range of defence mechanisms including fever, increase passage of white blood cells to infected tissues, the release of pro-inflammatory cytokines and vasodilation (Baumann and Gauldie, 1994; Suffredini et al., 1999; van Miert, 1995). Initiation of the acute phase response often causes undesirable effects on production, including impaired reproduction, decreased bone strength, anorexia, decreased growth rate and breakdown of skeletal muscle to fuel acute phase protein synthesis in the liver (Klasing et al., 1987; Xie et al., 2000; Barnes et al., 2002; Parker et al., 2002; Mireles et al., 2005). As selection for growth has had an effect on several other metabolic systems (McGary, 2003; Havenstein et al., 2003) it has been hypothesized that it has also adversely affected the birds' ability to deal with an immune challenge (Cheema et al. 2003), however research done in this area does not agree. Cheema et al. (2003) found that

a 2001 commercial broiler strain had an increased innate response but an impaired adaptive response when compared to 1957 Athens-Canadian random-bred strain. However previous work from our lab has found that both a 1957- and 1977- random-bred strains had greater total *in vitro* bactericidal activity than a modern commercial Ross 308 strain. In addition the 1957 strain had a greater heterophil oxidative burst response than either of the other strains suggesting a less of an innate response in the modern birds (Saunders-Blades et al., 2006 unpublished data).

The redirection of nutrients away from growth and towards immunity associated with the acute phase response has also been shown to affect the protein kinetics of the bird. Protein kinetics reflects a number of different measurements which can be used to determine the efficiency and rate at which protein is deposited, both at a whole body and a tissue level. Protein synthesis and breakdown are the rate at which amino acids are combined into protein and broken down and released by the tissue, respectively. Protein retention is the amount of amino acids retained by tissues; this can be positive or negative depending on whether the amount of protein synthesized is greater than that broken down or vice versa. Protein flux is the overall turnover of the amino acids. Together these measures can give useful information on general changes in protein metabolism (Kita, et al., 1993), genetics (Muramatsu et al., 1987) or environmental conditions (Aoyagi et al., 1988). Both protein synthesis and breakdown in the breast and liver have been shown to be increased when a bird undergoesan inflammatory challenge (Klasing and Austic, 1984a, 1984b). Selection for growth rate also affects protein kinetics in broiler chickens, decreasing protein degradation rates in the breast muscle (Tesseraud et al. 2000). As both growth potential and the inflammatory response can individually affect protein kinetics it

is possible that selection for growth has changed the way in which birds redistribute their resources, thus affecting protein metabolism in the process. This in turn may have affected the birds' requirements for several amino acids during and following an inflammatory response.

Lysine is an essential amino acid in poultry nutrition, and often the first or second limiting amino acid in grain-based diets (NRC, 1994). Dietary lysine requirements for growth (Labadan et al., 2001), egg production (Latshaw, 1976), and low growth rate (Coleman et al. 2003) have been thoroughly studied in poultry, however lysine's role in the immune system is less defined. As lysine requirements have been shown to decrease during an immune response suggesting dietary lysine may not play a large role lysine is an ideal starting point for determining amino acids requirements not only because of its importance in growth, but because of its limiting nature in the majority of grain based diets (NRC, 1994).

3.1.1 Project Objectives

The objectives of this study were to 1) confirm results from Chapter 2 using IAAO to determine lysine requirements during recovery from an acute phase response and 2) determine protein kinetics, including breakdown, synthesis and flux, and plasma amino acid concentrations during recovery from an acute phase response in 2 week old random-bred (R) and commercial chicks (C).

3.1.2 Hypotheses

Previously, Klasing and Barnes (1988) showed that lysine requirements were lower during an inflammatory response than that required for growth. However, a previous experiment showed that random-bred birds in which the acute phase response

had been stimulated with a bacterial LPS injection had higher lysine requirements than unstimulated controls during recovery phase (see Chapter 2). Therefore it was hypothesised that those results would be repeated in this experiment. Protein breakdown, synthesis, retention and flux were hypothesised to be higher in the commercial birds than the random-bred birds as both breakdown and synthesis have been shown to increase during an immune response at a tissue level (Tesseraud et al. 2000). Due to the increased degradation in the muscle and synthesis in the liver seen previously during an inflammation response, whole body protein synthesis, degradation and turnover were hypothesized to increase, while protein retention were expected to decrease, in LPS injected birds.

3.2 Materials and Methods

All procedures were approved by the University of Alberta animal care and use committee. Groups of birds (n=12 R and 12 C Ross 308 per week for 8 weeks) were raised in floor pens with ad libitum access to a nutritionally-complete starter feed until 10 d of age. At 10 or 11 d of age, birds underwent an adaptation period to adapt them to one of 7 diets, as previously described and analyzed in Chapter 2, consisting of either 6.07, 7.18, 8.38, 9.38, 10.49, 12.14 or 13.24 g/kg of dietary lysine (55% to 120% of NRC (1994) recommendation). At 12 or 13 d of age birds were injected with LPS and 12 hr post injection, an IAAO oxidation was performed as described previously (see Chapter 2). Individual bird and feed weights were recorded before injection and before and after each 4-hr oxidation study and birds were dosed with [1- ^{14}C] phenylalanine every 30 min during the study. Directly following the oxidation, blood was collected in heparinized

tubes via decapitation and for analysis of protein kinetics. Plasma was obtained by spinning blood at 3,000 rpm for 10 min at a constant temperature of 4°C and stored at -20°C until the analysis.

3.2.1 Feed Amino Acids

The experimental semi-purified diets were sampled and frozen until analyses were performed. Feed samples were analysed for amino acid composition using high performance liquid chromatography (HPLC) as previous described (Sedgwick et al. 1991).

3.2.2 Plasma Amino Acids

Plasma was thawed and 300 µl of each sample was transferred to a 1.5 ml microcentrifuge tube. Norleucine and 0.05 µCi/ml L - [U - ^{14}C] - leucine in 0.1 N HCL (40 µl) were added to the plasma as an internal standards. Protein in the plasma was precipitated by adding 1,000 µl of 1% triethylamine (TEA) solution, samples were vortexed and centrifuged at 12,000 rpm for 10 min. The supernatant was then transferred to plastic sample tubes, covered with parafilm and holes were placed in each cover to allow the escape of gases. The samples were then flash frozen in liquid nitrogen and freeze-dried over night. Once the samples were dry, 50 µl of a methanol:TEA: DDH_2O (20:20:60) solution was added . Samples were flash-frozen in liquid nitrogen for a second time and placed back in the freeze dryer. Once the samples were dry, derivitization was initiated by adding 50µl of a DDH_2O:TFA: phenylisothiocyanate (PITC):methanol solution (10:10:10:70). After 35 min, the reaction was stopped by placing the samples in liquid nitrogen, samples were then freeze-dried overnight. Once dry, the samples were reconstituted in 200 µl of phosphate buffer and spun at 12,000 rpm for 10 min. The

supernatant was then transferred to 0.15 ml micro centrifuge tubes and respun at 12,000 rpm for 10 min. The supernatant was then transferred to fresh 0.25 ml micro centrifuge tubes and spun again at 12,000 rpm for 10 min to remove the protein-bound amino acids from the sample. Standards were treated the same as the samples but did not contain plasma. The micro centrifuge tubes were then placed inside glass jackets and positioned on the HPLC for processing. The phenylalanine fraction of each sample was collected on the HPLC and the DPM of the ^{14}C in the sample was then counted using a liquid scintillation counter.

3.2.3 HPLC Buffers

Feed amino acids were run using a decreasing ratio of Buffer A:Buffer B; where buffer A was a mixture of anhydrous sodium acetate (33.34 g), HPLC-grade water (1,810 ml), methanol (180 ml) and tetrahydrasulfone (10 ml) adjusted to a pH of 7.4 and Buffer B was pure methanol. Plasma amino acids were run using a decreasing ratio of Buffer A:Buffer B; where buffer A was a mixture of sodium acetate (36 g), HPLC-grade water (4,000 ml), and acetonitrile (100 ml) adjusted to a pH of 6.55 and buffer B was a mixture of acetonitrile (1,800 ml), methanol (600 ml), and HPLC-grade water (1,600 ml).

3.2.4 Calculations for Plasma Kinetics

A number of whole-body protein kinetics variables were measured in the plasma for this experiment to allow calculation of protein retention, protein synthesis, protein breakdown, and protein flux. The calculations were as follows:

Recovery of [^{14}C] leucine (REC):

$$\text{REC (decays per min; DPM)} = \frac{\text{DPM [}^{14}\text{C] leucine in the sample}}{\text{(the pre-analyzed DPM of the [}^{14}\text{C] leucine used in the samples).}}$$

Specific radioactivity of the plasma (SRA):

SRA (DPM/mol) =

$$\frac{\text{DPM } [^{14}C] \text{ phenylalanine* Phenylalanine}(\mu mol/g) \text{ in the plasma sample}}{\text{REC(DPM)/.}}$$

Phenylalanine Flux:

$$\text{(Flux (mg/day)} = \frac{165.17\ \mu mol\ (\text{Infused (DPM/g/30min)}*1000)}{\text{SRA(DPM*g/ } \mu mol).} *48$$

Where 165.17 is the molar weight of phenyalanine and 48 is the number of half hour periods in a day.

Moles of phenylalanine oxidized:

Oxmol (mg/day) = flux (mg/day) * % [^{14}C] phenylalanine oxidized

Phenylalanine synthesis:

Synthesis (mg/day) = flux (mg/day) – oxmol (mg/day).

Phenylalanine Breakdown (breakdown):

Breakdown (mg/day) = flux (mg/day) – mg/d of phenylalanine in the feed.

Phenylalanine retention:

Retention (mg/day) = syn (mg/day) – breakdown(mg/day).

3.2.5 Statistical Analysis

Plasma kinetics, body weight, feed intake and amino acid plasma concentrations were analyzed using ANOVA in the GLM procedure of SAS with a significance level set at $P \leq 0.05$. A minimum of 4 birds were used to determine percent of phenylalanine dose oxidized per dietary treatment as previously determined (Leslie, 2003). Lysine requirements were determined using a linear plateau model in the non-linear procedure of SAS (Coleman et al. 2003). Percent dose of phenylalanine oxidized was regressed on dietary lysine content and the breakpoint was iteratively adjusted until a minimal residual

mean square was found. Variation around the means was determined using the non-linear procedure in SAS and treatments were compared using a T-test in the GLM procedure of SAS with a significance level set a $P \leq 0.05$.

3.3 Results and Discussion

3.3.1 Body Weight

The R birds had significantly lower BW than the C birds at hatch, before injection with LPS, before the oxidation and after the oxidation (Table 3-1). This was expected as R strains of birds have been shown to grow significantly slower than birds which have been selected for growth (Havenstein et al. 1994). Lipopolysaccharide injection did not have a significant effect on body weight ($P = 0.75$). This was surprising as in a previous experiment (see Chapter 2), LPS injection resulted in a reduction in BW of the C birds, but not the R birds. While the majority of studies have elicited responses betweenI and U birds, previous experiments have also shown inconsistent results concerning reductions in body weight following LPS injection. In broilers, Xie et al. (2000) found a decrease in body weight 12, 24 and 48 hr after injection; however Buyse et al. (2007) did not see any body weight change as a result of LPS injection from 1 to 10 d post injection. Differences in response to LPS between experiments could be a result of individual bird variation to LPS injection, although the exact mechanism is not known.

3.3.2 Lysine Requirements

The C birds had a significantly higher lysine requirement than the R birds (Figure 3-1) ($P \leq 0.0001$). The CU birds had a significantly higher lysine requirement than the CI ($P = 0.0126$) birds whereas the RU birds also had a significantly higher lysine

requirement than the RI birds ($P \leq 0.0001$). This differs from the results seen in Chapter 2 where LPS injection did not cause the C birds to differ in lysine requirement but the RI birds had a significantly higher requirement than the RU birds. Although the average body weight of the birds in Chapter 2 were slightly heavier than in this experiment (R: 119 g vs. 99 g C: 300 g vs. 237 g) this does not seem to be the reason for the differences as un-injected birds had similar requirements (C: 10.14 g/kg vs. 10.48 g/kg in this experiment; R: 8.32 g/kg vs. 8.28 g/kg in this experiment). The Ross 308 and the NRC (1994) requirements are stated at 1.2% (12 g/kg) and 1.1% (11 g/kg) of the diet respectively for birds of this age. While the CU birds show requirements similar to those reported by the NRC (1994), the random-bred birds' requirement was much lower but similar to what has been suggested by the NRC (1954), approximately 0.9% (9 g/kg). However, the injected birds varied greatly from these requirements in both strains as well as between studies (C: 11.95 g/kg vs. 7.45 g/kg in this experiment; R: 9.98 g/kg vs. 7.18 g/kg in this experiment).

3.3.3 Plasma Amino Acids

The R strain had higher plasma asparagine (P=0.0265) and lower glycine (P=0.0142) concentrations than the commercial strain (Table 3-2). The reason for the lower level of plasma glycine and higher levels of asparagine in the plasma of the random bred birds may be related to their use in the synthesis of uric acid (Christman and Mosier, 1929) and disposal of nitrogenous waste through the synthesis of ammonia (Wiggins et al. 1982), respectively. It is possible that the commercial diet given to the R strain did not as closely meet their requirements, causing more nitrogenous waste to be produced and

broken down (Pope et al., 2004). Plasma phenylalanine was higher in the I birds ($P<0.0001$) (Table 3-2). This could be in part due to higher phenylalanine concentrations in acute phase proteins than in muscle (Reeds et al., 1994). As the acute phase proteins start to break down during the recovery phase, less phenylalanine would be needed to rebuild skeletal muscle (Reeds et al., 1994), and more could appear in the plasma.

As plasma concentrations of lysine have been shown to increase with dietary levels (Corzo et al., 2003), the significant increase in plasma lysine seen with dietary lysine content was expected ($P<0.0001$; Table 3-3). In addition, plasma tyrosine concentrations of birds on the 95%, and 110% diets were significantly lower than the birds on the 55% and 65% diets. Tyrosine is synthesized from phenylalanine (Udenfriend and Cooper, 1952) and as lysine is likely limiting for protein synthesis in the lower lysine diets, less phenylalanine would be incorporated into muscle and more tyrosine may have been produced in the plasma. Plasma arginine concentration tended to decrease with an increase in dietary lysine ($P=0.070$) whereas the threonine concentration in the birds on the 75% diet was nearly significantly lower ($P=0.0723$) than birds on either the 55% or 65% diets (Table 3-3). The decreasing arginine concentration is most likely due to the well-known antagonism between lysine and arginine (Jones, 1964; Jones et al., 1967). This antagonism has been shown to decrease plasma arginine in chicks as lysine concentration increases (Jones, 1964). The reason for a trend to a lower plasma concentration of threonine in the 75% lysine diet as compared to the 55% and 65% diets is unknown.

3.3.4 Whole-Body Phenylalanine Kinetics

Whole-body phenylalanine kinetics were measured as a indicator of whole-body protein kinetics. The C birds had higher rates of protein flux, breakdown, synthesis and retention ($P \leq 0.0001$) (Table 3-4). This is most likely due to the higher growth rate and larger size of the C birds (Pym et al., 2004; Tesseraud et al. 2000).Lipopolysaccharide injection caused breakdown to significantly increase ($P = 0.050$) in the injected birds while both whole-body flux ($P = 0.065$) and synthesis ($P=0.061$) also showed a trend toward being significantly higher.

However, while whole-body protein breakdown seems to have increased with selection for growth, from previous research, this does not seem to be true within specific tissues. Fractional protein degradation rates were significantly lower in the breast muscle of birds selected for higher growth rate than unselected birds (Tesseraud et al. 2000). There is no information available on how recovery from immune response affects protein synthesis, breakdown, retention and flux in poultry and only limited information during an immune challenge. Following an injection with *E. coli* or sheep red blood cells, Klasing and Austic (1984b) did not see differences in protein breakdown in the liver. However, in broiler chicks injected with *E. coli*, a significant increase in protein turnover was seen in the breast muscle of broilers compared to U controls (Klasing and Austic 1984b). The increase in shown in a variety of tissues agrees with the overall increase in protein breakdown seen in this study.

Induction of the acute phase response in humans has been associated with an increase in transcription of several acute phase proteins (Birch and Schreiber, 1986). In poultry, many acute phase proteins such as hemopexin and α_1-acid glycoprotein are

known to increase significantly during the acute phase response (Adler et al., 2001). As protein kinetics were measured during recovery from the acute phase response, the increase in breakdown observed in the present study could be a result of acute phase proteins being catabolized as plasma levels return to normal. LPS injection in adult male Leghorns increased liver and plasma protein synthesis, however breast muscle protein synthesis was not affected (Barnes et al., 2002). Injection with *E. coli* in broiler chicks decreased the rate of protein synthesis in breast muscle and increased the rate in the liver (Klasing and Austic, 1984a). In humans, whole-body protein synthesis was increased during several types of acute disease (Long et al. 1977; Birkhahn et al., 1981; Long et al. 1981). In the current experiment, LPS injection resulted in a nearly significant increase whole body protein synthesis ($P<0.07$). This could be a result of measurements being taken during the recovery phase, or it could be indicative of compensatory growth, an increase in growth rate that often follows the short-term anorexia that accompanies acute inflammatory challenges (Beisel, 1977; Fitz-coy and Edgar, 1992). Although feed intake was recorded in this study, it was not deemed to be accurate due to spillage that could not be recovered as a result of cage design. Therefore, the degree of reduction in feed intake in this experiment could not be accurately determined.

The CI birds showed significantly greater protein synthesis, breakdown, and flux than their un-injected counterparts; however the RI birds did not differ significantly in any of the kinetic variables from the RU birds (Table 3-5). Although synthesis breakdown, and flux increased in the commercial birds due to LPS injection this did not seem to affect overall protein retention, suggesting a shift in the metabolic uses of the amino acids, possibly from immunity to growth, rather than just an increased protein

breakdown. As body weight remained similar between the CU and CI birds, this could suggest differences in metabolic regulation between the C and R birds during an immune response. As this experiment only examined protein kinetics at one point in time, it is possible that the recovery phase follows different time courses in R and C birds, such that recovery of normal protein metabolism happens at different time points for the two strains. This could be one reason as to why differences in kinetics where seen in the CI and CU birds but not between the RI and RU birds. As the R birds devote less of their total resources to growth than the C birds, more would theoretically be available for other systems such as immunity (Rauw et al., 1998). Therefore, during the recovery phase it is possible that the R birds would have fewer resources to shift back to growth providing less of a difference in kinetics between the RI and RU birds compared to their commercial counterparts. However, protein metabolism during recovery from an LPS injection has not been well studied in the avian system. Therefore, to determine mechanisms behind these changes and test these hypotheses, additional research into protein kinetics, including repeated sampling of the liver, breast muscle and plasma at different time points during the recovery phase, needs to be done.

It was surprising that lysine content of the diet did not significantly affect any of the protein kinetics parameters (Table 3-6), as increasing dietary lysine content has been previously shown to increase breakdown, synthesis and overall flux in both liver and muscle when lysine is fed excess of the requirement (Tesseraud et. al., 1996; Urdaneta-Rincon and Leeson, 2004). Broiler growth has also been shown to significantly increase with increasing dietary lysine up to the requirement (Tesseraud et al. 1999), suggesting there is also an increase in protein retention. However in the current study, no increase in

growth was seen as a result of increasing dietary lysine levels, perhaps also explaining the lack of change in whole body protein kinetics.

Results from this experiment agree with Klasing and Barnes (1988), who reported that LPS injection caused 3-week old broilers to have a lower lysine requirement than un-injected controls as determined by body weight gain and feed efficiency. A lower requirement during the acute phase response would fit, as acute phase proteins generally contain more tyrosine, phenylalanine, tryptophan, and less of the other amino acids as compared to skeletal muscle (Reeds, 1994). Since acute phase proteins are the main protein synthesized during an acute phase response (Klasing and Calvert, 1999), requirements could change to suit their production. However as the present measurements were made during the recovery phase, it would be expected that protein metabolism would be shifting away from acute phase protein production and back towards the production of skeletal muscle, thereby possibly increasing requirements for amino acids such as lysine which are essential for growth (Carew et al., 2005). It is possible that the birds had not yet started to shift protein synthesis back to skeletal muscle production and the increase in overall body protein kinetics seen in the injected chicks was still due to acute protein synthesis in the liver.

The results also may have been affected by the differences in the environmental temperature between the two experiments. Although exact environmental temperatures were not recorded, the experiment in Chapter 2 was conducted in the fall (September – November) whereas the current experiment was conducted in the summer (June – August). From the high level of condensation on the birds and the chambers as well as

from the temperature difference felt between the chambers and the room when the birds were removed, the ambient temperature in the current experiment seemed to be quite a bit higher than in Chapter 2 and may have been outside the birds thermoneutral zone. In addition mortality in the CI and CU birds was higher in the current experiment then in Chapter 2; however whether this was related to temperature was inconclusive. As a measure to reduce the temperature within the oxidation chambers the solid plastic tops of the metabolic chambers were replaced with plastic mesh until the actual oxidation.

One possibility is that the effects of LPS injection and temperature were an additive stress in the injected birds, thus increasing their lysine requirements. Lysine requirements have been shown to be both higher (Han and Baker, 1993) and lower (Corzo et al., 2003) than normal in birds raised under heat stressed conditions (37°C). LPS injection may cause problems with thermoregulation as the onset of fever is usually associated with an inflammatory response. As fever raises the hypothalamic set point, causing vasoconstriction and shunting blood away from the peripheral to the internal organs, this can severely limit or cease heat loss (Dinarello, 1996). Therefore coupled with high environmental temperatures it is possible the lack of heat loss could cause heat stress to occur at much lower temperatures than in a healthy bird. The effect of environmental temperature on the birds would also help to explain the lower body weights observed in the current experiment (R: 119g vs. 99g in this experiment C: 300g vs. 237g in this experiment). Birds under higher environmental temperatures have been shown to gain less weight than birds reared under normal conditions (Han and Baker, 1993; Alleman and Leclercq, 1997).

It is also possible that the birds in this study followed a different time course than the birds in Chapter 2 and that they were still in the acute phase when the measurements were made. This would account for the results agreeing with Klasing and Barnes (1988) as well as explain the discrepancy between the two experiments. However, a closer look at the protein turnover in both the liver and the breast muscle is needed to confirm this. As inconsistencies between experiments have been seen between a number of studies in a number of different variables measured it could be possible that these responses vary greatly between birds. Perhaps increasing the sample size, as only a minimum of four birds per dietary level were used in these experiments, would help to account for this variation.

3.4 Conclusions

In conclusion, this study showed that protein kinetics differed with both strain and LPS treatment whereas BW was lower in the CI birds compared to the CU birds but did not differ due to LPS injection in the R strain. Although all other variables except protein retention were increased by LPS injection in the C birds, the protein kinetics in the RI birds did not differ from the RU birds. These differences could be the result of metabolic differences in the rate of recovery from an immune response between the two strains, or could represent different time courses of recovery as measurements were only taken at one time point. Changes in lysine requirements and protein kinetics may also be indicative of compensatory growth following inflammation-induced anorexia; however, more research is needed before the mechanisms behind these changes can be fully defined. Determining how protein kinetics and amino acid requirements change during recovery from an immune challenge and as a result of selection for growth potential will

not only expand our knowledge of avian immunity but work towards applying this knowledge to our future nutritional and selection programs for poultry.

3.5 References

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Table 3-1: The Effect of Diet, LPS injection, and Strain on Body Weights of Random-bred and Commercial Birds

		Body Weight (g)			
		Hatch	Immediately Before Injection (12 or 13 days of age)	Before Oxidation (12 hours post injection)	After Oxidation (16 hours post injection)
[1]Strain	C	43.3[a]	249.3[a]	254.6[a]	265.9[a]
	R	34.0[b]	81.9[b]	82.9[b]	86.0[b]
[2]LPS Treatment	I	38.4	163.4	165.6	172.9
	U	38.9	167.8	171.9	178.9
[3]Diet (% of NRC requirements for lysine)	55	40.0	160.4	163.7	168.1
	65	38.6	154.1	155.2	160.6
	75	38.3	163.9	165.3	173.8
	85	38.2	165.8	168.0	178.2
	95	37.5	166.3	175.5	183.4
	110	39.1	168.4	170.1	177.9
	120	38.7	180.1	183.2	189.5
Pooled SEM		0.58	7.24	7.15	7.45
P value	Strain	<0.0001	<0.0001	<0.0001	<0.0001
	LPS Treatment	0.5505	0.4780	0.3208	0.3684
	Diet	0.7932	0.4388	0.2917	0.2765
	Strain*LPS Treatment	0.7055	0.9188	0.7873	0.9192
	Strain*Diet	0.3588	0.6386	0.6143	0.4609
	LPS Treatment*Diet	0.5403	0.7543	0.4409	0.5747
	LPS Treatment*Diet*Strain	0.6346	0.7232	0.4596	0.5099

[a,b]Means with differing superscripts within a main effect represent values which differ significantly (P > 0.05).
[1]Means represent 59-68 birds per treatment; LPS = Lipopolysaccharide; C = commercial strain; R = random-bred strain
[2]Means represent 59-68 birds per treatment; LPS Treat = lipopolysaccharide treatment; I = birds injected with LPS; U = birds not injected with LPS;
[3]Means represent a minimum of 16 birds per treatment. Diets contain 6.07, 7.18, 8.38, 9.38, 10.49, 12.14 or 13.24 g/kg of dietary lysine (55% to 120% of NRC (1994) recommendation)

Table 3-2: Effect of LPS Injection and Strain on Plasma Amino Acid Concentrations in 2 Week old Broiler Chicks

Variable	Strain[1]		LPS Treat[2]		Pooled SEM	P Value[3]	
	C	R	I	U		Strain	LPS Treat
ASP	0.555[b]	0.704[a]	0.644[a]	0.620[a]	0.122	0.027	0.884
GLU	0.411	0.433	0.425	0.420	0.013	0.329	0.839
SER	2.340	2.557	2.530	2.364	0.759	0.253	0.300
GLY	0.796[a]	0.628[b]	0.719[a]	0.694[a]	0.147	0.014	0.846
HIS	0.315	0.277	0.298	0.292	0.045	0.378	0.937
THR	2.166	2.465	2.461	2.175	1.184	0.142	0.155
ALA	1.004	0.975	1.030	0.946	0.116	0.651	0.165
ARG	0.424	0.429	0.445	0.408	0.018	0.823	0.131
PRO	0.773	0.774	0.795	0.753	0.0774	0.940	0.399
TYR	0.075	0.078	0.079	0.074	0.001	0.664	0.228
VAL	0.277	0.284	0.286	0.275	0.015	0.752	0.575
MET	0.485	0.496	0.510	0.471	0.048	0.923	0.332
CYS	1.220	1.309	1.288	1.244	0.120	0.281	0.527
ILE	0.159	0.152	0.143	0.168	0.008	0.653	0.135
LEU	0.303	0.321	0.316	0.308	0.001	0.200	0.568
PHE	0.135[a]	0.128[a]	0.146[a]	0.116[b]	0.001	0.330	<0.0001
LYS	0.245	0.219	0.214	0.249	0.025	0.265	0.215

[1]Means with differing superscripts within the strain columns represent values which differ significantly; means represent 55-57 birds per treatment C = commercial strain; R = random-bred strain

[2]Means with differing superscripts within the LPS treatment columns represent values which differ significantly; means represent 55-57 birds per treatment; I =birds injected with LPS; U = birds not injected with LPS

[3] There were no significant interactions.

Table 3-3: The Effect of Diet on Plasma Amino Acid Concentrations in 2 Week old Broiler Chicks

Amino Acid	Diet (% of NRC requirements for lysine)[3]							Pooled SEM	P Value
	55	65	75	85	95	110	120		Diet
ASP	0.468	0.649	0.681	0.591	0.730	0.711	0.583	0.122	0.420
GLU	0.419	0.413	0.438	0.408	0.427	0.413	0.442	0.013	0.976
SER	2.302	2.778	2.412	2.458	2.420	2.076	2.586	0.759	0.452
GLY	0.630	0.664	0.649	0.644	0.918	0.620	0.794	0.147	0.172
HIS	0.328	0.246	0.424	0.288	0.266	0.307	0.232	0.045	0.205
THR	2.750	2.753	1.758	2.089	2.458	2.073	2.252	1.184	0.072
ALA	0.960	1.041	1.006	0.952	0.928	1.015	1.010	0.116	0.949
ARG	0.494	0.4664	0.429	0.423	0.433	0.35	0.370	0.018	0.070
PRO	0.769	0.819	0.805	0.786	0.822	0.657	0.720	0.0774	0.635
TYR	0.087^{ab}	0.091^{a}	0.076^{abc}	0.081^{abc}	0.066^{c}	0.064^{c}	0.067^{bc}	0.001	0.031
VAL	0.300	0.301	0.287	0.309	0.250	0.257	0.253	0.015	0.623
MET	0.441	0.572	0.400	0.477	0.528	0.464	0.520	0.048	0.325
CYS	1.181	1.239	1.161	1.376	1.359	1.097	1.394	0.120	0.139
ILE	0.158	0.156	0.188	0.173	0.152	0.129	0.123	0.008	0.406
LEU	0.331	0.349	0.311	0.314	0.293	0.293	0.281	0.001	0.217
PHE	0.138	0.122	0.151	0.129	0.129	0.138	0.115	0.001	0.136
LYS	0.118^{a}	0.140^{a}	0.209^{ab}	0.256^{b}	0.203^{ab}	0.358^{c}	0.381^{c}	0.025	<0.0001

[1]Means with differing superscripts represent values which differ significantly; means represent 11-16 birds per treatment
[2] There were no significant interactions
[3] Diets contain 6.07, 7.18, 8.38, 9.38, 10.49, 12.14 or 13.24 g/kg of dietary lysine (55% to 120% of NRC (1994) recommendation)

Table 3-4: The Effect of Strain and LPS Treatment on Plasma Protein Kinetics in 2 Week old Commercial and Random-bred Broiler Chicks

Variable[1]	Strain[2]		LPS Treat[3]		Pooled SEM	P value[4]	
	C	R	I	U		Strain	LPS Treat
BW (kg)	0.237[a]	0.099[b]	0.161	0.169	0.009	<0.0001	0.750
Protein							
Flux (mg/day)	1985.9[a]	765.2[b]	1410.4	1251.9[a]	85.59	<0.0001	0.065
Synthesis (mg/day)	1929.4	753.6[b]	1378.6	1218.9[a]	83.11	<0.0001	0.061
Retention (mg/day)	718.6[a]	343.7[b]	508.1	526.2[a]	31.20	<0.0001	0.862
Breakdown (mg/day)	1224.9[a]	413.8[b]	889.6	692.7[b]	78.83	<0.0001	0.050

[1] BW = body weight; FI = feed intake

[2]Means with differing superscripts within the strain columns represent values which differ significantly; means represent 55-57 birds per treatment LPS = Lipopolysaccharide; C = commercial strain; R = random-bred strain

[3]Means with differing superscripts within the LPS treatment columns represent values which differ significantly; means represent 55-57 birds per treatment; LPS Treat = lipopolysaccharide treatment; I = birds injected with LPS; U = birds not injected with LPS;

[4] There were no significant interactions.

Table 3-5: A Comparison of Protein Kinetics Between LPS Injected and Control Birds in Commercial and Random-bred Strains

Variable[1]		BW (kg)	FI (mg)	Protein Flux (mg/day)	Protein Synthesis (mg/day)	Protein Breakdown (mg/day)	Protein Retention (mg/day)
Strain/LPS Treatment	CI	0.236	1964.4	2192.9[a]	2135.1[a]	1441.3[a]	730.2
	CU	0.240	1849.8	2135.1[b]	1745.0[b]	1039.4[b]	707.2
	RI	0.099	800.6	800.7	788.9	486.0	320.3
	RU	0.103	901.7	724.6	712.7	349.6	360.7
Pooled SEM		0.009	77.9	85.6	83.11	78.83	31.20
P value	CI vs CU	0.8316	0.5114	0.0343	0.0322	0.0487	0.7672
	RI vs RU	0.8111	0.5474	0.6602	0.6506	0.4719	0.5675

[1] BW = body weight; FI = feed intake; C = commercial strain; R = random-bred strain; I = birds injected with LPS; U = birds not injected with LPS

[2]Means with differing superscripts within a column and strain represent values which differ significantly; means represent 55-57 birds per treatment LPS = Lipopolysaccharide; C = commercial strain; R = random-bred strain

Table 3-6: The Effect of Diet on Plasma Protein Kinetics in 2 Week old Commercial and Random-bred Broiler Chicks

Variable[1]	Diet (g diet)[3]							Pooled SEM	[3]P Value Diet
	55	65	75	85	95	110	120		
BW (kg)	0.189	0.154	0.167	0.171	0.152	0.170	0.180	0.009	0.652
FI (mg/oxidation)	1412	1253	1492	1114	1663	1401	1158	77.88	0.196
Protein (mg/day)									
Synthesis	1538.9	1182.1	1472.6	1375.0	1263.1	1170.4	1088.3	83.11	0.524
Retention	463.5	495.6	516.9	433.2	666.1	498.7	460.0	31.20	0.203
Breakdown	1075.4	686.5	956.0	941.8	597.0	671.7	618.5	78.83	0.485
Flux	1590.1	1210.0	1510.8	1407.3	1291.6	1195.3	1112.6	85.59	0.490

[1] BW = body weight; FI = feed intake
[2]Means with differing superscripts represent values which differ significantly; means represent 11-16 birds per treatment
[3] Diets contain 6.07, 7.18, 8.38, 9.38, 10.49, 12.14 or 13.24 g/kg of dietary lysine (55% to 120% of NRC (1994) recommendation)
[3] There were no significant interactions.

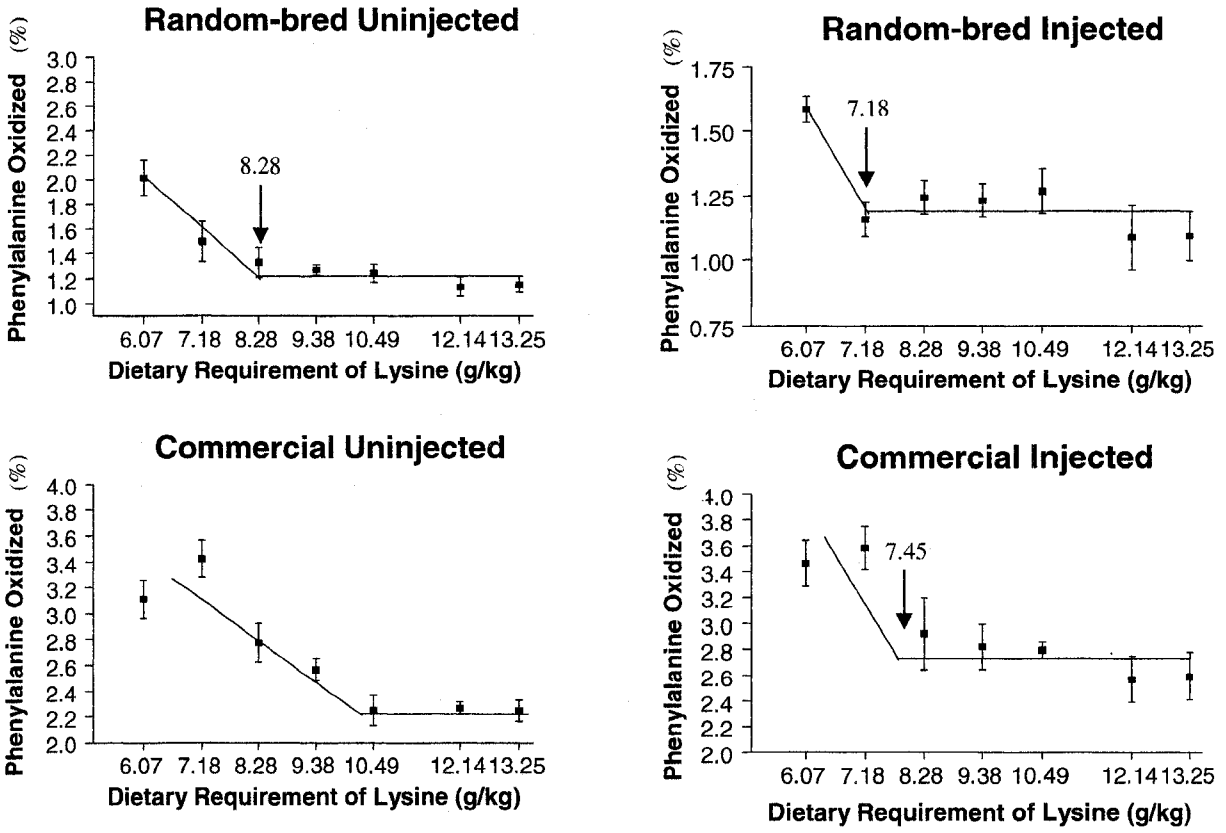

Figure 3-1: Lysine Requirements as Determined by IAAO of 2-Week Old Random–bred and Commercial Broilers. Injected birds were injected intra-abdominally at 11 or 12 d of age with 2 ml of a 100 μg/ml solution of *S. typhimurium* LPS. Oxidations occurred 12 hours following injection, body weights were taken immediately prior to the oxidation. Arrows represent the lysine requirement in g/kg.

4. SYNTHESIS

Commercial broilers have been continuously selected for growth potential over the past several decades. This selection has led to adverse changes in a number of different systems, including skeletal abnormalities (Havenstein et al., 1994), reproductive problems (McGary et al., 2003) and an increase in mortality rate due to metabolic diseases such as ascites (Julian, 2000). Selection for growth may also have had an impact on the immune system of broilers; however, less research has been conducted in this area.

The immune system is generally divided in to two main components, the innate system and the adaptive system. The innate system is present in the bird from hatch and provides a general protection against a variety of different innate structures, common to a number of different organisms. The adaptive system offers a more specific protection to the bird by building defences against specific invaders and keeping a "memory" of that antigen to provide a faster more efficient response to a second challenge. However, the adaptive system takes time to mature, approximately a week in newly hatched chicks, and needs to be directed to harmful organisms via the innate system. Therefore, any unfavourable changes to the innate immune system resulting from selection for growth have the potential to drastically affect production throughout the bird's lifetime.

The acute phase response is one manifestation of the innate immune system. Activation of the acute phase response results in a number of defence mechanisms including fever, anorexia, and redirection of nutrients from skeletal muscle to the liver in order to form acute phase proteins. This redirection of nutrients away from growth and towards inflammation has also been shown to affect the protein kinetics, including increased protein synthesis and breakdown in both the breast and liver (Klasing and

Austic, 1984a, 1984b). As priorities for nutrients shift, the amounts of each amino acid needed by various systems also change, altering the requirements of the bird certain amino acids.

The objectives of the current studies were to determine if selection for growth potential has had an effect on several parameters in broiler chickens recovering from an acute phase response. Lysine requirements, protein kinetics, body weight and plasma amino acid concentrations were measured in a line of birds random-bred since 1957 (R) and a commercial Ross 308 (C) strain. A time-course of the acute phase response was also measured by body temperature changes over an 18 hr period following experimental induction of an inflammatory response. An inflammatory response was induced in half the birds of each strain by an intra-abdominal injection of LPS. It was hypothesized, in the time course study that temperature would increase with LPS injection as this effect has been reported in numerous studies in both mammals and birds. The R birds had a higher temperature throughout the experiment than the C birds; both the R and C birds injected with LPS (RI, CI) displayed higher temperatures than the un-injected birds (RU, CU) by 4 hr post-injection. Temperatures started to decrease around 12 hr post- injection and all birds were fully recovered by 18 hr post-injection. This study confirmed that IP injection of LPS caused an increase in body temperature in broilers. Knowing the time course of the LPS induced acute phase response in broilers will not only increase our knowledge of the innate immune response, but allow experiments to be planned at specific phases within this response, such as the recovery phase.

Lysine requirements were measured during recovery from the acute phase response (between 12 and 16 hr post-injection) in both R and C strains using the indicator

amino acid oxidation method (IAAO) in two different experiments. It was hypothesized in Chapter 2 that lysine requirements would be lower in the CI and RI birds compared to the CU and RU birds. During the acute phase response lysine requirements have shown to decrease (Klasing and Barnes, 1988) and considering the birds had not yet fully recovered, requirements were expected to be lower than for growth. As reported in Chapter 2, contrary to the hypothesis, the lysine requirement of the RI birds was increased whereas the CI birds' requirements were not affected by LPS injection. However, despite the higher lysine requirement of the R birds, only the C birds' body weight gain was decreased by LPS injection. As these measurements were taken during the recovery phase it seemed a reasonable explanation that the increased requirement may be a result of the RI birds experiencing compensatory growth as this has been shown to occur in birds following infection (Newcombe et al., 1992) as well as increased amino acid requirements (Plavnik and Hurwitz, 1989). The lack of effect in the CI birds could be due to differences in time course of recovery or individual bird variation.

The experiment conducted in Chapter 3 attempted to verify the IAAO results from Chapter 2 and it was hypothesized that lysine requirements and body weights for both strains would be similar to those found in Chapter 2. Protein breakdown, synthesis, retention and flux were also determined in order to establish whole body protein kinetics during recovery from the acute phase response. Protein kinetics, specifically breakdown, synthesis and flux, were expected to increase as the birds would most likely be slowing down production of acute phase proteins in the liver while increasing skeletal muscle turnover.

However when requirements were determined in Chapter 3, both CI and RI birds displayed lower lysine requirements than their un-injected counterparts. In addition, neither strain of birds showed changes in body weight in response to LPS injection. Despite the lack of body weight response to LPS injection, differences were seen in protein kinetics between the injected and uninjected birds in both strains. Protein synthesis, breakdown, retention and flux were all higher in the C birds compared to the R strain. Protein breakdown was significantly increased in the injected birds, whereas both whole-body flux ($P = 0.065$) and synthesis ($P=0.061$) also showed a trend toward being significantly higher due to LPS injection. The CI birds had a significantly higher protein synthesis, breakdown and flux than the CU birds, whereas the RI birds protein kinetics did not differ from those of the RU birds.

During the recovery phase, protein synthesis, breakdown, retention and flux were all higher in the C birds compared to the R strain. This was expected as the C birds had a significantly larger body weight and growth rate than the R birds. Looking at LPS injection within strains, the higher breakdown, synthesis and flux of the CI birds without a change in retention suggests an inflammation-induced shift in protein metabolism. This could be caused by a larger redirection of nutrients in the C birds, caused by the breaking down of acute phase proteins and the rebuilding of muscle at the time point at which the blood was taken. As no differences in any of the protein kinetics were seen in the R birds due to LPS injection, it could also suggest that these birds may have a different time course for recovery with redirection of nutrients happening later or earlier than at the specific time point measured. It is also possible that this redirection in the R birds was of a smaller magnitude, making it harder to detect than in the C line.

The lack of an effect on body weight reported in Chapter 3 was unexpected as a multitude of literature has shown body weight to decrease with LPS injection (Parmentier et al., 1998; Webel et al., 1998; Mireles et al., 2005). However, both the lysine requirement and the protein turnover data of injected birds were significantly different from the un-injected controls suggesting that the LPS injection did successfully induce an inflammatory response in the birds. Whereas the un-injected birds had similar requirements between the two experiments (C: 10.48 vs 10.12 g/kg; R: 8.32 vs 8.28 g/kg) the requirements of the injected birds were much lower in the second experiment (C: 11.95 vs 7.45 g/kg; R: 9.98 vs 7.18 g/kg). Body weight results were also variable, both between and within the experiments. In Chapter 2, LPS injection decreased body weight gain of the C birds but not the R birds whereas in Chapter 3, body weight was not significantly affected by LPS injection in either strain. These results seem to suggest LPS injection can cause highly variable responses during the recovery phase of an acute phase response on requirements and body weight gain. While the vast majority of studies show a drop in body weight associated with an LPS induced inflammatory response (Parmentier et al., 1998; Webel et al., 1998; Mireles et al., 2005), there have been iinstances in which no change in body weight between LPS injected birds and uninjected controls have been reported (Xie et al., 2000). In addition, on rare occasions, body temperature has shown to deviate from the expected response. For example, Leshchinsky and Klasing (2001) saw a decrease in body temperature of broilers rather than the normal increase associated with intra-abdominal injection of LPS. Due to this variation, in order to get a true depiction of the general pattern, a larger sample size per strain x treatment x diet level may be needed when looking at the responses to an inflammatory response.

These variable responses may also have been caused by the differences in environmental temperature between the two chapters. While exact temperatures were not recorded, Chapter 2 was conducted in the fall (September – November) while the Chapter 3 was conducted in the summer (June – August). Based on the condensation in the boxes and on the birds as well as the actual temperature difference felt between the environment and boxes when the birds were removed the ambient temperature seemed to be higher during the experiments performed in Chapter 3 compared to those in Chapter 2. Lysine requirements have been shown to be higher then normal requirements in birds raised under high temperatures (37°C) (Han and Baker, 1993). As an initiation of an inflammatory response is known to include development of a fever this may explain why birds could have been outside there thermoneutral zone during the experiments conducted in Chapter 3. Fever causes an increase in the hypothalamic set point, initiating vasodilation and redirecting blood from the periphery to the internal organs (Dinarello et al., 1996). This causes the amount of heat dissipated by the animal to decrease and a high environmental temperature coupled with the inability to dissipate heat may explain why injected birds would become heat stressed at lower temperatures than their un-injected counter parts, resulting in decreased requirements whereas the un-injected birds were unaffected and maintained the same requirements as seen in Chapter 2. This could also help to explain the lower body weight of the birds in Chapter 3 as birds under higher environmental temperatures have been shown to gain less weight then birds reared under normal conditions (Han and Baker, 1993; Alleman and Leclercq, 1997). The differential effects of LPS seen on lysine requirement and body weight between Chapter 2 and Chapter 3, implies that the responses to LPS are not always consistent, and may be

somewhat dependent on environmental conditions. Therefore for future research, ensuring thermoneutrality during future experiments may be prudent when examining amino acid requirements

As concerns regarding the use of antibiotics grow, new methods of dealing with the effects on production will be essential. During recovery from the acute phase response in humans, large amounts of body protein can be retained compared to healthy subjects, suggesting an increase in protein synthesis (Kurpad, 2006) and making the recovery phase a possible target for applying alternate measures to help encourage growth in immune-challenged birds. By increasing our knowledge about the changes that occur during recovery from an acute phase response, as well as our understanding of how selection for growth affects it, we can use this knowledge not only to direct future selection goals but also to develop new ways to reduce the effect of inflammatory challenges on production.

Results seem to change from study to study for all parameters tested, this may suggest that environment and individual variation among birds may play a large role in the manifestation of the innate immune response. Studies examining different time points during the recovery phase may help to clarify if the R birds follow a different time frame for recovery than the C birds. Measurements of protein kinetics within individual tissues, such as the liver and the breast, may help us to get a better view of how resources are redistributed, which may help in understanding changes in amino acid requirements. As consumer concerns grow about the use of prophylactic antibiotics in animal industries, new ways to deal with the constant immune challenges without the use of antibiotics will have to be discovered. Expanding our knowledge of the avian immune system will

become invaluable as these changes occur and may allow us to explore nutritional alternatives.

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