Nutritional applications of arginine functionality to support broiler chicken health and performance

by

Annalise Grace Anderson

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Approved by

Dr. Samuel J. Rochell, Chair, Associate Professor of Poultry Science
Dr. William A. Dozier III, Professor of Poultry Science
Dr. Rüdiger Hauck, Associate Professor of Poultry Science and Pathobiology
Dr. Marko Rudar, Assistant Professor of Animal Sciences
Dr. Haruka Wada, Outside reader, Associate Professor of Biological Sciences

ABSTRACT

Four experiments were conducted to evaluate the effects of dietary dArg:dLys ratio on the performance and health of broilers subjected to different immunological, environmental, and pathogenic stress models. Experiment 1 used intradermal (i.d.) lipopolysaccharide (**LPS**) injections at 40 d to stimulate the innate immune response, and the local (tissue) and systemic (blood) inflammatory responses were evaluated at 0, 6, 24, and 48 h post-injection. Experiments 2 and 3 used a cyclic heat stress (**HS**) model in Ross 708 and Cobb 500 genotypes during the finisher phase to mimic the environmental challenges experienced by broilers in temperate zones during the summer and in tropical regions year-round. Experiment 4 utilized oral gavages of a 10-fold dose of a live coccidiosis vaccine at 15 d followed by ~10^7 CFU *Clostridium perfringens* at 19 and 20 d to replicate enteric challenge similar to what may be encountered by broilers in the field. In each experiment, dietary treatments included multiple (5 to 7) dietary dArg:dLys ratios that ranged from below to well above the current breeder recommendations. Linear and quadratic contrasts as well as regression analyses were used to evaluate dose-responses of dependent variables across increasing dArg:dLys ratios. In experiment 1, the local and systemic inflammatory response to i.d. injected LPS was dominated by an increase in heterophils (**H**) and a drop or no effect in lymphocyte (**L**) populations. On average, there was a linear increase in tissue and blood H with increasing Arg that also translated to a higher H:L ratio. Likely, this response was mediated by nitric oxide (**NO**), which increased linearly with increasing dArg:dLys ratio at 6 h post-injection. Concentration of alpha-1-acid glycoprotein (**AGP**) in the plasma and relative expression of IL-1β, IL-6, and IL-8 mRNA levels in tissue also responded in a dose-dependent manner to dietary Arg. In experiments 2 and 3, feed conversation ratio (**FCR)** was linearly improved during the finisher phase with increasing dArg:dLys ratio in both genotypes,

but body weight gain (**BWG**) and feed intake were not affected. At 32, 38, and 46 d post-hatch, HS increased cloacal temperatures by approximately 1.6ºC, and dArg:dLys influenced body temperature dose-dependently in the Ross genotype only and not the Cobb. For processing characteristics, there were linear increases in chilled carcass and tender weights and yields and quadratic responses for breast fillet and total breast weights and yields in the Ross genotype. In the Cobb genotype, there was a quadratic response for fat pad weight and yield and linear increases in thigh and drum weights. In experiment 4, the enteric challenge significantly impaired growth performance during all periods, increased plasma NO and AGP concentration, and decreased ileal secretory IgA secretion. During the challenge recovery period (22 to 29 d), FCR of challenged birds improved linearly with increasing dArg:dLys, and increasing dArg:dLys ratio quadratically improved BWG and FCR of challenged birds during the grower phase and cumulatively. Dietary Arg did not significantly affect 22 d plasma NO or AGP concentration in this trial. For processing characteristics, feeding 1.29 dArg:dLys improved chilled carcass yield compared to birds fed 1.05 dArg:dLys. Overall, the results of these experiments indicate that increased dArg:dLys ratio beyond the current recommendations of 1.05 to 1.10 may be beneficial for improving broiler performance and health. This is likely due to the secondary metabolic functions of Arg, including synthesis of NO, that has diverse roles in supporting immunity and vasodilation.

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I. INTRODUCTION

When using least cost diet formulation for commercial broiler production, the practice of specifying a minimum level of dietary crude protein (Kidd et al., 2000) has effectively been replaced by formulating on a digestible amino acid (**AA**) basis. The inclusion of crystalline forms of AA allows nutritionists greater flexibility in achieving ideal AA ratios (Han and Baker, 1994; Emmert and Baker, 1997; Baker et al., 2002), which increases nutrient utilization, lowers feed costs, reduces excess nitrogen excretion into the environment, and improves litter quality (Kidd et al., 2000; Nahm, 2007; Van Harn et al., 2019; Greenhalgh et al., 2020; Adhikari et al., 2023).

The 1st limiting AA for poultry is methionine, which gained acceptance in its synthetic form for inclusion in commercial feeds in the 1960s (Kidd et al., 2013). Lysine, the 2nd limiting AA, was next to be adopted in the 1970s (Kidd et al., 2013). Research throughout the 1990s provided insights into threonine requirements, the 3rd limiting AA, and crystalline threonine allowed nutritionists to continually improve their ability to meet the birds' requirements for essential AAs (Kidd et al., 2013). As each AA is provided in the diet closer to its expected requirement level based on performance under optimal conditions, understanding how factors that may influence these requirements becomes more important to minimize potential deficiencies.

Depending on the ingredients used, the 4th limiting AA in USA-based poultry diets is usually arginine (**Arg**), valine, or isoleucine (Adhikari et al., 2023). Fortunately, feed-grade versions of these AA have recently become more widely available and economically feasible for use in commercial diet formulation (Kidd et al., 2013; Adhikari et al., 2023). Arginine, specifically, is not only a protein constituent but also a precursor to many important molecules

including nitric oxide, creatine, polyamines, and proline (Castro and Kim, 2020). Thus, it is considered a "functional AA" due to its diverse roles beyond protein synthesis and accretion in immunity, thermoregulatory vasodilation, and gastrointestinal health (Taylor and Bishop, 1993; Wu, 2010; Tan et al., 2014; Liang et al., 2018; Castro et al., 2020). Consequently, particularly in scenarios of immune or environmental challenge, it has been hypothesized that the Arg requirements of broilers to support these secondary functions may not be the same as those traditionally used to optimize growth performance and meat yield. Thus, the experiments in this dissertation were designed to evaluate the nutritional applications of Arg functionality to support broiler chicken health and performance during different stress states. Specifically, experiment 1 used intradermal (i.d.) lipopolysaccharide injections to stimulate the innate inflammatory response, and the local (tissue) and systemic (blood) inflammatory responses were subsequently evaluated at 0, 6, 24, and 48 h post-injection. Experiments 2 and 3 used a cyclic heat stress model during the finisher phase to mimic the environmental challenges experienced by broilers in temperate zones during the summer and in tropical regions year-round. Finally, experiment 4 utilized oral gavages of a live coccidiosis vaccine followed by ~10^7 CFU *Clostridium perfringens* to replicate enteric challenge similar to what may be encountered by broilers in the field. In each trial, dietary treatments included multiple (5 to 7) dietary dArg:dLys ratios that ranged from below to well above the current breeder recommendations, and Arg responses were evaluated across titration levels to determine the effects on growth performance, meat yield, and indicators of Arg metabolism.

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II. LITERATURE REVIEW

OVERVIEW

L-Arginine (**Arg**) was first isolated in 1886 from lupine seedling extracts by German chemists E. Schulze and E. Steiger (Wu, 2021). Arginine is a basic amino acid (**AA**) with the chemical formula $C_6H_{14}N_4O_2$, and as such, Arg has the highest nitrogen (**N**) content (32.16%) of all 20 proteinogenic AAs (Wu, 2021). The side chain of Arg consists of a three-carbon aliphatic straight-chain ending in a guanidinium group that has a large p*K*a (12.48), and therefore, is always protonated and positively charged at physiological pH (Numata, 2021).

The endogenous synthesis of Arg by mammals was first determined in the classic nutrition studies by W. C. Rose and his colleagues at the University of Illinois in the 1930s (Carter and Coon, 1995; Wu, 2021). Although de novo synthesis of Arg is possible in ureotelic animals (animals that excrete N waste as urea), it is considered a semi-essential AA, meaning that in certain conditions like growth or severe physiological stress, endogenous synthesis of Arg may be insufficient to meet its demand (Cynober et al., 1995). In mammals, Arg is synthesized from L-citrulline (**Cit**) via argininosuccinate by the enzymes argininosuccinate synthase (EC 6.3.4.5) and argininosuccinate lyase (EC 4.3.2.1) as part of the urea cycle (Cynober et al., 1995). These enzymes are found predominantly in the liver, but as the hepatic arginase (EC 3.5.3.1) activity is high, the net production of Arg from the liver into circulation from this process is low (Cynober et al., 1995). Instead, endogenously synthesized Arg is mostly derived from Cit in the kidney, which is obtained from glutamine and glutamate metabolism in the intestine (Cynober et al., 1995).

Arginine metabolism for uricotelic animals (animals that excrete N waste as uric acid) such as birds, is different. In chickens, various enzymes of the urea cycle do not exist in the liver and are instead found in only moderate amounts in the kidney (Tamir and Ratner, 1963). In addition, Tamir and Ratner (1963) showed that chickens lack the activity of carbamoyl-phosphate synthetase 1 (EC 6.3.4.16) in all tissues, preventing the de novo synthesis of Cit from L-ornithine (**Orn**) and ammonia (Tamir and Ratner, 1963; Castro and Kim, 2020). Further yet, Wu et al. (1995) found no expression of P-5-C synthase (EC 1.5.1.2) and only very low activity of ornithine aminotransferase (EC 2.6.1.13) in chicken enterocytes, indicating that intestinal conversion of glutamate into P-5-C and P-5-C into Orn occurs at a very low rate (Fernandes and Murakami, 2010). It is for these reasons that chickens can synthesize some quantity of Arg from Cit in the diet but not from Orn (Tamir and Ratner, 1963; Fernandes and Murakami, 2010), and that Arg is considered an essential AA for poultry. It is also notable that the arginase activity in the kidney of chickens is very high (nearly 30 times higher than in the liver and twice that of the rat kidney) (Tamir and Ratner, 1963), meaning that Arg supplied in the diet must be sufficient enough to satisfy both the needs of the animal and also replace that degraded in the kidney. Kidney arginase activity can be further increased with a high dietary content of lysine (**Lys**) (Nesheim, 1968).

Fortunately for nutritionists, common feed ingredients used for poultry such as soybean meal and meat and bone meal contain a reasonable amount of Arg (Arg is 3.2% and 3.7% of the total AA in these feed ingredients, respectively), with gelatin (7.7%), peanut meal (5.7%), and feather meal (5.7%) containing even higher amounts (Li et al., 2011). In modern commercial poultry diets, Arg could be the 4^{th}, 5^{th}, or 6^{th} limiting AA depending on the ingredient profile, as certain ingredients like corn DDGS, bakery meal, sorghum, or corn gluten meal can be

particularly low in Arg (Li et al., 2011; Adhikari et al., 2023). However, feed-grade crystalline L-arginine has recently become more widely available and economically feasible for use in commercial poultry diets, providing nutritionists increased flexibility to optimize Arg and other AA requirements in diets using synthetic essential AAs and without increasing crude protein (Adhikari et al., 2023).

Historically, research surrounding synthetic AAs has primarily focused on optimizing protein synthesis and accretion (Castro and Kim, 2020), specifically meat weight and yield in broiler chickens. Although this is still a main focus of today, there is a growing body of literature dedicated to investigating the secondary functions of AA on metabolism. This is because it has been recognized that certain AA are precursors to other important molecules involved in essential processes like immune defense, cell signaling, and antioxidant responses (Castro and Kim, 2020), and thus, the requirements for those AA under certain conditions may be different than those traditionally used to optimize growth performance and meat yield (Kidd, 2004). Because of their significant impact on the growth, development, and survival of animals, Wu (2010) termed these AA "functional" AA, which includes Arg (Castro and Kim, 2020). Taken together, it has become essential to define the dietary Arg requirements of broilers in different states of immune or environmental challenges to adequately support these secondary functions and optimize broiler nutrition and health, as they may not be the same as those needed to support productive performance.

ARGININE-RELATED METABOLITES

Aside from being a protein constituent, Arg is the metabolic precursor to important molecules like nitric oxide (**NO**), polyamines, proline (**Pro**), and creatine (**Crn**) (Fernandes and

Murakami, 2010). It is also a potent secretagogue for the secretion of anabolic hormones like insulin, growth hormone (**GH**), and insulin-like growth factor-1 (**IGF-1**) (Fernandes and Murakami, 2010; Yu et al., 2018). The biochemical pathways by which Arg is associated with these metabolites as well as their functions are detailed herein, and diagrams visually detailing the pathways of Arg metabolism can be found in reviews by Fernandes and Murakami (2010) and Castro and Kim (2020). As would be assumed, each of the metabolites related to Arg define the secondary functionality of this AA in their own way.

Arg Catabolism. Arginine has two direct degradative pathways for its catabolism (Cynober et al., 1995). The first is by nitric oxide synthase (**NOS**) enzymes (EC 1.14.13.39) that produce Cit and NO (Morris and Billiar, 1994; Fernandes and Murakami, 2010), and the second is by arginase (EC 3.5.3.1), which catalyzes the formation of urea and the generation of Orn (Cynober et al., 1995). From the latter reaction, polyamines can be formed by the decarboxylation of Orn to putrescine (**Put**), which can then be irreversibly converted to spermine (**Spm**) and spermidine (**Spd**) by the relevant enzymes (Gonzalez-Esquerra and Leeson, 2006; Fernandes and Murakami, 2010). Alternatively, Orn can be converted via P-5-C to Pro, despite the enzyme activity for this reaction in chickens being very low (Wu et al., 1995; Wu et al., 2011). Arginine can also be catabolized for the biosynthesis of Crn in the process of two reactions. The guanidine group of Arg is first transferred to glycine to form guanidinoacetate (**GAA**), and then GAA is methylated by S-adenosylmethionine (a substrate produced from methionine) to form S-adenosylhomocysteine and Crn (Fernandes and Murakami, 2010).

Nitric Oxide. Nitric oxide is a highly reactive biological N free radical that has many diverse physiological functions (Snyder and Bredt, 1992; Butler et al., 1995; Fernandes and Murakami, 2010). It is utilized throughout the animal kingdom as a signaling molecule and as

toxic agent between cells, and can act as a complex regulator of immune and inflammatory function through multiple pathways (Fang and Li, 1989; Wink et al., 1996; Coleman, 2001; Guo et al., 2015). It also has roles in neurotransmission, endothelium-dependent vasodilation, regulation of blood pressure, inhibition of platelet aggregation, and an inducer or suppressor of apoptosis (Snyder and Bredt, 1992; Coleman, 2001). One molecule of L-Arg produces one molecule of NO, as the N atom of the latter is derived from the guanidino group of the Arg side chain (Coleman, 2001).

There are three types of NOS enzymes that convert L-Arg and molecular oxygen to NO. Endothelial (**eNOS**) and neuronal (**nNOS**) are constitutively expressed, while inducible (**iNOS**) is expressed only in activated cells (Morris and Billiar, 1994; Coleman, 2001). The constitutive forms generate NO in response to a calcium (**Ca**) signal, which rapidly activates these NOS to produce a rapid and pulse-like NO signal to responding cells such as neurons or smooth muscle cells (Coleman, 2001). Alternatively, iNOS activity is induced by immunological stimuli including bacterial lipopolysaccharide (**LPS**) or cytokines such as IL-1 or TNF-α, and generates larger quantities of NO after a lag period of several hours that reflects time taken to synthesize mRNA and protein (Coleman, 2001). Inducible NOS generates NO in much higher quantities than the constitutive forms (nano rather than picomolar), and this level of synthesis is maintained for days or longer depending on the time that NOS is present in the cells or tissue (Coleman, 2001).

The signaling action of NO can be by both direct and indirect action. At lower concentrations of NO like those generated by eNOS and nNOS, NO directly interacts with positively charged metal ions in proteins such as the iron atom in the heme moiety of guanylyl cyclase (Coleman, 2001). In this case, cGMP-dependent protein kinases are produced that can

mediate relaxation in smooth muscle cells, neuronal transmission in neurons, and inhibition of platelet aggregation, for example (Butler et al., 1995; Coleman, 2001). At higher concentrations, however, the actions of NO are mainly indirect. At high concentrations NO is extremely unstable, and under aerobic conditions is rapidly oxidized to form reactive nitrogen oxide species. Reactive nitrogen oxide species can chemically modify enzymes, signaling proteins and transcription factors, which produce long-term cellular effects (Coleman, 2001). In addition, high concentrations of NO paired with high oxidative stress can cause NO to react with superoxide anion (O_2^-) to form the highly toxic peroxynitrite ($OONO^-$), which is responsible for most of the severe toxic effects of NO (Butler et al., 1995; Wink et al., 1996).

Polyamines. The three primary types of polyamines are Spe, Spd, and Put (Sagar et al., 2021). They have several important functions in the body including cell proliferation and differentiation, gene regulation, cell signaling, and apoptosis (Pegg, 2016). Thus, maintenance of normal polyamine content is essential for normal animal growth and development. In fact, depletion of polyamines not only inhibits cell growth but also enhances apoptosis (Nilsson et al., 2000; Moinard, 2005). Rapidly dividing cells and tissues with a high proliferation rate contain higher amounts of polyamines, which can benefit gastrointestinal tract development and maintenance of barrier function, for example (Löser et al., 1999; Tan et al., 2024).

In chickens, polyamines are synthesized in the kidneys, which are likely the main source of polyamines in the blood and extra-renal tissues (Furukawa et al., 2021). The concentration of polyamines in the plasma of broilers is 20 to 100 times higher than that of mammals to support their high rates of protein synthesis and rapid growth (Baker, 2009). In fact, there are age-dependent changes in renal polyamine synthesis and tissue concentration that may be correlated to synthesis rate for skeletal protein (Furukawa et al., 2021). There is also evidence in piglets to

support that polyamines can also promote intestinal mucosal repair after injury (Wang et al., 2015).

Proline. Proline is a predominant AA in collagen proteins, which are major extracellular components in connective tissues like skin, tendon, cartilage, vessels of the vascular system, and bone (Li and Wu, 2018). Proline can also have roles in antioxidative reactions (Kaul et al., 2008). Thus, adequate provision of Pro is essential for maximal growth performance of animals and can be particularly beneficial in regard to wound healing and tissue repair (Graber and Baker, 1973; Barbul, 2008; Li and Wu, 2018). However, it should be noted that in chickens endogenous synthesis of Pro from Orn in enterocytes is low (only 4% that of cells from pigs) with no synthesis of Pro via the P-5-C pathway (Wu et al., 1995); thus, growing chickens can synthesize only about 90% of the Pro it needs for maximal growth (Graber and Baker, 1973). As synthetic Pro and gelatin (an abundant source of Pro) are expensive (Li and Wu, 2018), their inclusion in practical animal rations is limited and thus alternative dietary precursor sources like Arg could be of particular value.

Creatine. Creatine is involved in cellular energy metabolism through ATP regeneration, and has a direct role in the accretion of protein by improving the availability of ATP for myosin (Portocarero and Braun, 2021). More specifically, Crn functions as a carrier and a rapidly mobilizable high-energy phosphate reserve in cells that have a high and variable energy demand (i.e., skeletal muscle and the brain) to regenerate ATP (DeGroot et al., 2018; Portocarero and Braun, 2021). This can be particularly important in fast-growing species like broiler chickens, that have a higher need for Crn to support the rapid rate of muscle tissue accretion (Brosnan et al., 2009). There is evidence to suggest that dietary Arg (and GAA) can influence both serum and muscle total Crn in broiler chickens (DeGroot et al., 2018), and in fact, Chamruspollert et al.

(2002) reported that as Arg is a precursor of Crn and cannot be synthesized de novo, Crn balance in birds is normally dependent on dietary Arg (Portocarero and Braun, 2021). Crystalline Crn is neither commercially available nor stable enough to withstand the standard feed manufacturing process (Baker, 2009; DeGroot et al., 2018); thus, supplying precursor molecules in the diet like Arg or GAA instead of Crn itself can be particularly advantageous.

Secretagogue for Hormones. Arginine has a secretagogue activity by which it stimulates the release of anabolic hormones like GH and IGF-1, likely via a decrease in somatostatinergic tone (Cochard et al., 1997; Oh et al., 2017; Yu et al., 2018). Therefore, Arg has the potential to influence protein synthesis and animal growth (Kwak et al., 1999) in this way. In pigs, Cochard et al. (1997) observed a significant increase in plasma GH when Arg was intravenously infused into the veins of 35 d piglets, and Kim et al. (2004) reported that the addition of 0.4% Arg to the diets of young piglets increased GH and insulin concentrations by 24-27% compared to the control. In chickens, Yu et al. (2018) showed that serum levels of IGF-1 were significantly increased in 42 d Lohmann Brown layers fed increasing concentrations of Arg throughout the rearing period, and Xu et al. (2018) reported that serum concentrations of GH, IGF-1, and insulin in broilers, as well as body weight, increased quadratically at 21 d with increasing concentration of Arg in the diet.

ARGININE AND THE INFLAMMATORY RESPONSE

In commercial poultry production, the health of broiler chickens relies majorly on the nonspecific innate immune system given that their short production period occurs mostly while the adaptive immune system is developing (Hamal et al., 2006; Simon et al., 2014; French et al., 2020). Studies have shown that Arg can have roles in the regulation of innate immunity (Lee et

al., 2002; Tan et al., 2014a), which includes the inflammatory response, most likely through being the metabolic precursor for NO (Hassan et al., 2021). Not only can NO act as a toxic defense molecule towards infectious organisms, but it can also be a potent vasodilator and regulator of the functional activity, growth, and death of many immune and inflammatory cell types like macrophages, T lymphocytes, and natural killer cells (Coleman, 2001; Kröncke et al., 2001; Chen et al., 2008). It is also possible that Arg influences the immune response by increasing the activity and expression of antioxidant enzyme families like glutathione peroxidase and superoxide dismutase that alleviate oxidative stress (Duan et al., 2015; Liang et al., 2018). Regardless of the mechanisms, potential enhancement or optimization of the innate immune system with nutritional interventions like Arg are of particular interest to support the health, welfare, and production efficiency of chickens.

To artificially stimulate the inflammatory response in a research setting, injections of lipopolysaccharide (**LPS**; endotoxin) may be utilized. Lipopolysaccharide is a naturally occurring component on the cell walls of gram-negative bacteria, and is a potent stimulator of inflammation through the Toll-like receptor 4 (**TLR4**) expressed on the surface of macrophages and other leukocytes (Dil and Qureshi, 2002; Kogut et al., 2005; Tan et al., 2014a; French et al., 2020). After injection, these leukocytes become activated to produce and release vasoactive mediators, cytokines, and chemokines that can lead to inflammation (French et al., 2020). Lipopolysaccharide models have been used to study the effects of dietary Arg on the inflammatory response of chickens, and are detailed herein.

Takahashi et al. (1999) fed broilers an Arg-deficient, sufficient, or excess diet for 7 d and then intraperitoneally injected them with LPS. This study was intended to determine how dietary Arg may affect plasma NO during the acute-phase response. The authors found that at 6 and 10 h

post-LPS injection, peak plasma NO concentrations were obtained, which returned to pre-injection levels by 24 h. However, there were dose-dependent statistical differences in plasma NO concentration during peak NO production, where increased dietary Arg consistently resulted in higher NO. The production of NO also coincided with plasma concentration of alpha-1-acid glycoprotein (**AGP**), which is a positive acute phase substance. These results indicated that dietary Arg concentration can have proportional effects on the production of NO after LPS injection, additionally affecting down-stream outcomes of the inflammatory response (AGP).

In a broader evaluation of how dietary Arg may affect the innate immune response, Tan et al. (2014a) fed broiler chickens 1.05, 1.42 and 1.90% total Arg concentrations (0.73, 0.97 and 1.30% total Arg:Lys) and injected them with either LPS or saline at 48 h intervals between 14 and 21 d of age. The LPS injections suppressed the percentage of $CD11^+$ and B cells in the spleen (as a percentage of splenic lymphocytes), increased the relative mRNA expression of pro-inflammatory cytokines (IL-1β and IL-6) in the spleen and cecal tonsils, and increased the expression of TLR4 in the spleen. However, there were significant main effects of Arg in that higher Arg concentrations linearly reduced the percentages of splenic $Bu1^+$, $CD11^+$, and $CD14^+$ cells, linearly decreased the expression of IL-1β in the spleen, and linearly down-regulated the expression of TLR4 in the spleen and cecal tonsils. It was concluded from this experiment that Arg can alleviate systemic inflammation, potentially through the suppression of the TLR4 pathway.

Using layer breeds, Lieboldt et al. (2017) fed 12 wk old cockerels 70, 100, and 200% of the recommended Arg supply to determine if Arg could affect the hematological and febrile response of these chickens in 48 h post-LPS injection. It was determined that Arg had no effect on the rectal body temperature during this acute-phase response, but that Arg-deficient cockerels

had higher total leukocyte counts than birds fed the adequate and excessive Arg. Interestingly, both deficient and surplus Arg tended to cause a higher ratio of heterophils to lymphocytes (**H:L**) in the blood, and as a higher H:L ratio is associated with a higher degree of stress in healthy birds (Gross and Siegel, 1983), it was suggested that dietary imbalances of Arg may intensify immunological and metabolic stress in sick chickens. With a similar experimental design, Lieboldt et al. (2015) found that insufficient Arg reduced body weight gain (**BWG**) and increased the relative weights of the liver and pancreas after LPS injection in layer pullets, and that increasing dietary Arg also increased plasma concentrations of Arg, Cit, Orn, and 3-methyl-histidine.

Other than LPS, inflammatory responses can also be experimentally initiated using different methods. For instance, Lee et al. (2002) used infectious bronchitis virus (**IBV**) to inoculate broilers fed a moderately-deficient, adequate, and high concentration of Arg in order to study the influence of dietary Arg on leukocyte responses after infection. At 1 and 7 d post-inoculation (**dpi**), samples of peripheral blood and respiratory lavage were collected. It was found that dietary Arg significantly increased the percentage and absolute number of heterophils, decreased the percentage of lymphocytes, and increased the H:L ratio in the peripheral blood at 1 dpi. The percentage of heterophils in the respiratory lavage was also increased by Arg at 1 dpi. By 7 dpi, all of these differences had equalized, but the percentage of $CD8^+$ respiratory lavage cells in birds fed the deficient diet was lower than those from birds fed the adequate or high level of Arg. As heterophils are the first leukocyte to accumulate at the site of inflammation and infiltrate rapidly, within h (French et al., 2020), this study demonstrates strong evidence that dietary Arg can influence the early distribution of leukocyte subpopulations after IBV inoculation in a target tissue for the infection.

Zhang et al. (2020) evaluated effects of Arg supplementation on the inflammatory response and intestinal injury of broilers after a challenge with *Salmonella enterica* serovar Typhimurium. Broilers were fed either 1.04% or 1.24% total Arg:Lys and either unchallenged or challenged with *S. typhimurium*. It was found that dietary Arg supplementation could largely alleviate the intestinal histopathological damage caused by the challenge, which was determined by jejunal histopathological scores. It was also observed that *S. typhimurium* challenge elevated levels of inflammatory parameters in the serum, but that Arg supplementation was able to decrease the IL-1β, IL-8, and LPS-induced TNF-α factor homolog concentration compared to the control. In addition, Arg reduced the IL-8 mRNA expression in the jejunum in challenged birds. Thus, Arg supplementation in this experiment was proven to ameliorate the inflammatory response and alleviate intestinal mucosal impairment after *S. typhimurium* challenge in broilers.

Regardless of the model used, these aforementioned studies repeatedly demonstrate that dietary Arg supplementation can significantly influence elements of the inflammatory response in chickens. Although changes to intestinal health, acute-phase protein production, leukocyte population responses, and inflammatory cytokine production are shown herein, these measurements are often single time-point assessments and thus lack data to characterize the Arg effect on the full time course (initiation and resolution) of the acute inflammatory response. In addition, as NO is often recognized to be the primary mediator of the immunomodulatory effect of Arg (Hassan et al., 2021), it would make sense to include these analyses more frequently in future experiments. However, it should be noted from Takahashi et al. (1999) that these data may be fairly time sensitive.

ARGININE AND HEAT STRESS

Heat stress (**HS**) occurs when the amount of heat energy produced by an animal exceeds the amount of energy dissipating from its body (Lara and Rostagno, 2013). Poultry can be particularly sensitive to HS due to their lack of sweat glands and a limited unfeathered body surface area to facilitate heat loss (Zhang et al., 2017b). Modern broilers may be even more susceptible due to their high metabolic rates (Settar et al., 1999; Syafwan et al., 2011). In fact, Teyssier et al. (2022a) reported a 16-45% reduction in feed intake (**FI**) and a 20-67% reduction in BWG in modern broilers subjected to cyclic and constant HS models. As HS has been proven to cause deleterious effects on bird behavior, physiology, welfare, gut health, and growth performance, nutritional interventions like Arg that may ameliorate some of these negative effects can be particularly useful (Brugaletta et al., 2022; Teyssier et al., 2022b).

Arginine may benefit poultry experiencing HS through its role as a precursor for NO and polyamines. Specifically, NO can influence both central and peripheral thermoregulatory vasodilation (Taylor and Bishop, 1993; Canini et al., 2001; Uyanga et al., 2021) and reduce oxidative injury caused by high ambient temperatures (Rubbo et al., 1996; Atakisi et al., 2009; Tan et al., 2010; Shan et al., 2013). In its thermoregulatory role, NO can centrally influence sympathetic tone by influencing various brain sites, and peripherally it can reduce vascular smooth muscle tone and arteriolar vasodilation (Branco et al., 2014). Both mechanisms can result in an increase in the blood flow to the skin, delivering heat to the body surface dissipated into the environment (Branco et al., 2014). Additionally, as HS can cause damage to the intestinal epithelia, polyamines may help to maintain gastrointestinal barrier function and benefit mucosal integrity and repair (Yun et al., 2020; Tan et al., 2024).

Early studies to evaluate dietary Arg concentration for broiler chickens during HS were published in the 1990s, with Balnave and Oliva (1991) reporting that the digestibility of Arg in diets was significantly decreased at an ambient temperature of 30°C compared to 18°C or increased compared to 21°C, suggesting that HS may alter the absorption of Arg in the intestine. Mendes et al. (1997) reported that an increased Arg:Lys ratio may be beneficial to enhance bird performance and processing characteristics during HS, but in this study claimed it could not be confirmed if this was due to Arg per se or a nonspecific N response. Therefore, Brake et al. (1998) published a series of several experiments to specifically evaluate broiler responses to Arg:Lys during HS, and concluded that the ideal AA balance for broilers can in fact change with ambient temperature, as discussed below.

In one experiment, broilers were reared from 20 to 41 d at either constant 31°C (HS) or 20°C (thermoneutral; **TN**), and fed diets containing either 1.09% or 1.36% total Arg:Lys. It was determined that HS decreased BWG and FI as expected, but also that the ratio of 1.36% Arg:Lys increased BWG under 20°C but the trend was only numeric at 31°C. In addition, feed conversion ratio (**FCR**) was improved by 1.36% Arg:Lys at 31°C but not 20°C. A second experiment using a cyclic model of HS also resulted in a significant improvement in FCR in broilers reared in hot temperatures fed a higher (1.37% versus 1.10%) total Arg:Lys ratio but no change in performance for birds in the TN environment. A third experiment in which broilers were fed 0.94, 1.08, 1.25, and 1.39% total Arg:Lys from 42 to 55 d also showed a significant temperature by diet interaction for FCR where FCR linearly decreased with increasing Arg:Lys under HS, but not for TN birds. Thus, these results provide strong evidence that performance benefits of Arg may be sensitive to rearing temperature.

More recently, the number of studies evaluating dietary Arg and HS in broilers has grown. For growth performance, the majority report that increasing dietary Arg concentrations in broilers subjected to HS experience improvements in FCR only (Mendes et al., 1997; Brake et al., 1998; Lee et al., 2024; Oliveira et al., 2024). However, some also report improvements in BWG (Nassar et al., 2022; Abd El-Maged and Desoky, 2023), while others report no performance benefits from Arg supplementation at all (Brugaletta et al., 2023). This variability in results may be due to dietary Arg concentrations and number of levels chosen for evaluation, variation in the type and severity of the HS challenge model, dietary sodium chloride concentration, and differences between broiler genetics, among others.

Outside of growth performance, these aforementioned studies also report that higher dietary Arg concentration during HS may result in higher villus height and lower crypt depth in the intestine (Lee et al., 2024), increased levels of Crn in the plasma, liver, and breast muscle (Brugaletta et al., 2023), higher concentration of red blood cells and hemoglobin and percentage of packed cell volume in the blood (Nassar et al., 2022), increased relative weights of carcass dressing, liver, spleen, bursa, and thymus (Nassar et al., 2022), and a reduction of total cholesterol content (Abd El-Maged and Desoky, 2023; Oliveira et al., 2024). The mechanisms by which Arg exerts these effects are likely different between each case, but Lee et al. (2024) hypothesized that measured improvement in intestinal physiology in their experiment was due to Arg being a metabolic precursor to the polyamines.

Broilers are not the only livestock species shown to benefit from increasing dietary Arg concentration during HS challenges. Zhu et al. (2014) found that 5 g per kg L-Arg supplementation significantly improved 0 to 49 d feed efficiency of White Pekin ducks experiencing cyclic HS by 3.7% compared to ducks fed the basal diet (13.7 g/kg starter, 11.5

g/kg grower), and also increased the relative weight of liver by 7.8% at 21 d. Yun et al. (2020) reported increasing Arg supplementation to weaning pig diets under hot season conditions linearly improved average daily gain and feed efficiency. And Laspiur and Trottier (2001) showed that 1.73% Arg tended to reduce the weight loss of lactating sows and increase her feed efficiency compared to 0.96% Arg (control) diets during 29°C HS. In vitro, 6 mmol/L L-Arg in culture medium reduced HS injury to bovine intestinal cells by improving the antioxidant and inflammatory response, suggesting that L-Arg could be an effective dietary addition for dairy cows to protect them from adverse effects of HS on the intestine (Dou et al., 2023). The results of these studies show that supplementation of Arg to animal diets during HS can be beneficial not only for birds, for which Arg is an essential AA, but also for animals in which Arg can be largely synthesized endogenously and is considered only a conditionally essential AA.

ARGININE AND ENTERIC DISEASE

Enteric diseases cause significant economic losses to the poultry industry each year, with coccidiosis alone estimated to cost the global industry approximately US$3 billion annually (Dalloul and Lillehoj, 2006; Blake et al., 2020). Coccidiosis infections are caused by parasites from the genus *Eimeria*, which complete part of their life cycle in host epithelial cells causing intestinal inflammation, malabsorption, excess production of mucus, and decreased growth performance (Chapman et al., 2014; Rodgers et al., 2015). These consequences also make coccidiosis one of the main predisposing factors for the development of necrotic enteritis (**NE**), which is induced by an overgrowth of the opportunistic bacteria *Clostridium perfringens* that causes further mucosal tissue necrosis, hemorrhaging, reduced intestinal integrity, dehydration, and in some cases, high (30%) mortality (Gholamiandehkordi et al., 2007; Van Immerseel et al.,

2009; Adhikari et al., 2020; Lorenzoni, 2023). With increasing consumer demand for antibiotic-free poultry products (Adhikari et al., 2020), manipulating the dietary Arg concentration could be part of an effective strategy for the control of enteric diseases in chickens.

There are a number of studies that show dietary Arg supplementation above the recommended values could benefit broiler growth performance and gut morphological characteristics during coccidiosis. Castro et al. (2020) fed Cobb 500 broilers 5 increasing digestible Arg:Lys ratios (ranging from 0.88 to 1.22% dArg:dLys; below to above the breeder recommendation) and challenged them with mixed *Eimeria* spp. at 12 d. In this experiment, the authors reported that BWG and FI increased linearly with increasing concentrations of Arg from 0 to 14 dpi, quadratically decreased intestinal permeability at 5 dpi, and dose-dependently increased crypt depth in the duodenum (linear) and jejunum (quadratic) at 6 dpi. The benefits of Arg to the health of the intestine were again thought to be due to Arg being a precursor to the polyamines, as Arg supplementation increased the intestinal concentration of polyamines and improved the cellular proliferation and intestinal repair after ischemia damage in rats (Raul et al., 1995). A study by Tan et al. (2014b) found that feeding coccidial-challenged broiler chickens increasing concentrations of Arg linearly decreased TLR4 mRNA expression level in the jejunum, again indicating that Arg may also promote intestinal health by attenuating intestinal mucosal inflammation via suppression of the TLR4 signaling pathway, similar to the results of Tan et al. (2014a) using LPS.

Another study by Laika and Jahanian (2017) supplied Ross 308 broilers with Arg at 100, 105, and 110% of the standard industry recommendation and challenged broilers with mixed *Eimeria* spp. from 16 to 20 d. These authors found that Arg supplementation improved FCR during the grower (14 to 28 d) and finisher (28 to 42 d) periods, increased villi height to crypt

depth ratio, decreased the muscular layer thickness of the jejunum, and reduced fecal oocyst count. Izadi Yazdanabadi et al. (2020) fed Ross 308 broilers 85, 100, 125, and 150% of their recommended dietary Arg requirement and challenged them with mixed *Eimeria* spp. at 21 d. Birds fed higher Arg concentrations (125 and 150%) had higher ADG in the grower (11 to 24 d) and finisher (25 to 42 d) periods than those fed lower levels (85 and 100%), and those fed Arg levels of 100 to 150% had lower FCR than those fed 85%. In addition, the concentration of serum NO increased linearly at 42 d as dietary Arg concentration increased. This finding supports the fact that NO can be an important mediator for innate and acquired immunity during coccidiosis infections, specifically as a toxic substance to some parasites (Allen and Fetterer, 2002), and may be a prominent mechanism by which Arg improves broiler performance during enteric disease.

There are a lesser number of studies that evaluate the effect of Arg supplementation in broilers subjected to NE, and performance benefits to broilers are less consistent than in coccidiosis. In a series of two experiments, Fathima et al. (2024a,b) evaluated growth performance, intestinal health, and immune responses of broilers supplemented with Arg during a NE challenge. In the first experiment (Fathima et al., 2024a), Cobb 500 male broilers were fed a basal diet ± NE challenge or 125% Arg ± NE challenge. The authors found that Arg supplementation decreased FI during the grower phase (14 to 21 d), but the corresponding improvement in FCR was only numeric. Arginine supplementation was also found to downregulate the 21 d relative gene expression of the iNOS gene in the cecal tonsils of infected birds, but it did not have a significant effect on the permeability of the intestine, jejunal lesion scores after challenge, or relative gene expression of tight junction proteins in the jejunum. In the second experiment (Fathima et al., 2024b), Cobb 500 male broilers were fed a basal diet ± NE

challenge, 125% Arg + NE challenge, or 135% Arg + NE challenge. Again, Arg supplementation had no influence on growth performance except that 135% Arg increased FI over 0 to 35 d compared to the basal + NE treatment, but 135% Arg + NE did result in a higher $CD8^+$: $CD4^+$ ratio in the spleen at 28 d and lower $CD8^+$: $CD4^+$ ratio in the cecal tonsils at 21 d compared to the basal + NE. At 21 d, 125% Arg had a 10-fold increase in the relative mRNA expression of iNOS compared to the basal + NE treatment, but not at 28 or 35 d. It was concluded from these experiments that Arg supplementation may improve broiler immunity during NE infections, and thus could be used in conjunction with other nutritional interventions to combat NE.

Zhang et al. (2017a) and Zhang et al. (2019) specifically investigated the effect of Arg on the intestinal inflammatory response induced in broilers by *Eimeria* spp. and *C. perfringens*. In the first study (Zhang et al., 2017a), broilers were fed a basal diet (1.05% total Arg:Lys) or a high Arg diet (1.57% total Arg:Lys) and challenged with *Eimeria* and *C. perfringens*. As expected, the challenge induced intestinal damage as indicated by higher paracellular permeability in the ileum, higher numbers of *C. perfringens* in the cecal digesta, and decreased relative mRNA expression levels of jejunal claudin-1, which were all alleviated by Arg supplementation. Regardless of challenge, Arg also increased relative mRNA expression of IL-10, IFN-γ, and NOD-1 in the intestine, implying that Arg can promote the innate immune response and protect the intestinal mucosa. In vitro, these authors showed that NO had bacteriostatic activity against *C. perfringens*, which provided evidence that Arg can inhibit the overgrowth of *C. perfringens* directly through the production of NO. Furthermore, Zhang et al. (2019) reported that 1.24% total Arg:Lys (compared to 1.04%) fed to NE-challenged birds reduced the relative mRNA expression of jejunal IL-6, JAK-1, JAK-3, STAT-1, and STAT-6,

decreased the NE lesion scores and histopathological injury in the jejunum, and reduced the amount of *C. perfringens* enumerated from the liver. Regardless of challenge, Arg also reduced the relative mRNA expression of inflammatory cytokines (IL-1β, IFN-γ, IL-10, and TGF-β3) in the jejunum and tended to reduce jejunal mucosal iNOS activity. It was concluded that Arg can also down regulate the JAK-STAT signaling pathway to attenuate the inflammatory response during *C. perfringens* infection, exerting positive effects on the broiler intestine.

In addition to supplemental Arg, recent work has also investigated the effects of dietary Cit supplementation for the control of NE in broiler chickens (Dao et al., 2022a,b,c). The rationale for this is that Arg is subjected to extensive splanchnic first pass metabolism (in mice, up to 70%) whereas Cit is not (Agarwal et al., 2019; Uyanga et al., 2023), and thus, dietary Cit (after being recycled to Arg de novo) has actually been shown to be more effective in increasing blood Arg concentration and NO production than Arg itself in many animal species (Schwedhelm et al., 2008; Lassala et al., 2009; Wijnands et al., 2012; Agarwal et al., 2019). Specifically in chickens, Uyanga et al. (2020) found that dietary Cit increased serum Arg and plasma NO concentrations in laying hens, and Uyanga et al. (2023) showed that dietary Cit supplementation elevated kidney and liver NO concentration, plasma eNOS activity, and expression of intestinal tight junction proteins (claudin-1 and occludin) higher than that of Arg itself during Arg deficiency in broilers.

Thus, to explore potential benefits of dietary Cit during enteric disease, Dao et al. (2022a,b,c) challenged male Ross 308 broilers with subclinical NE and fed them a standard protein (**SP**) diet, reduced crude protein (**RP;** 2% lower protein) diet, RP + supplemental Arg (103% of Arg in RP and also 103% of the Arg requirement), or RP + supplemental Cit equivalent to the supplemental Arg. Regarding growth performance, the key take-away was that

the overall 0 to 35 d FCR of broilers fed the RP + Cit diet was significantly lower than that of the SP diet, but that of the RP and RP + Arg diet were intermediate and not different (Dao et al., 2022a). The same statistical ranking occurred for 35 d breast meat yield (Dao et al., 2022a). For jejunal morphology, RP + Cit increased villus height compared to those fed the SP or RP diet and increased the villus height to crypt depth ratio compared to the RP diet. However, RP + Arg was again intermediate and not statistically different from either treatment (Dao et al., 2022b). There were also no significant differences in the relative mRNA expression of jejunal tight junction proteins or inflammatory-related genes among the RP diets (Dao et al., 2022c). As a result, the efficacy of Cit to replace Arg in broiler diets during enteric infection requires further validation, perhaps using different disease models or dietary concentrations.

Overall, the results of these studies reviewed herein show that dietary Arg supplementation can certainly have benefits on the productive performance, inflammatory response, and intestinal health of boilers experiencing enteric disease stress. It appears that as the effects of the enteric challenge become increasingly detrimental, Arg alone may not be sufficient to significantly reverse the effect of the challenge on broiler growth performance. However, perhaps in these instances the importance of Arg supply shifts more towards the support of the immune response. This continues to validate the use of dietary Arg in feed formulation during enteric disease, in conjunction with other nutritional interventions.

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III. INFLUENCE OF DIETARY ARGININE RATIO ON LOCAL AND SYSTEMIC INFLAMMATORY RESPONSES TO LIPOPOLYSACCHARIDE IN BROILERS

ABSTRACT

The objective of this study was to evaluate the effects of dietary Arg ratio on the local and systemic, innate inflammatory responses of the broiler chicken. A total of 280 male chicks from a Cobb 500 line were fed a common diet for 2 wk before being fed 1 of 5 target dietary dArg:dLys ratios (0.77, 0.91, 1.05, 1.19, or 1.33) across grower (14 to 28 d) and finisher (28 to 42 d) phases. Birds were reared in battery cages and immune parameters evaluated over 39 to 42 d using a growing feather (**GF**) model. Specifically, the GF-pulps of broilers were intradermally (**i.d.**) injected with 10 μL phosphate buffered saline (**PBS**; 2 birds/diet, 12 GF/bird) as a vehicle control or 10 μL of lipopolysaccharide (**LPS**; 6 birds/diet, 12 GF/bird; 1 μg LPS/GF) to stimulate an inflammatory response. Blood and GF were collected at 0 (before), 6, 24, and 48 h after GF injection. GF-pulp and blood cell suspensions were analyzed for indicators of the local and systemic inflammatory response, including GF-pulp and blood leukocyte profiles, relative GF-pulp cytokine mRNA expression, and plasma alpha-1-acid glycoprotein (**AGP**) and nitric oxide (**NO**) concentrations. Data were analyzed by 2-way repeated measures ANOVA for blood or 2-way ANOVA for GF, with time and diet as main effects. Linear and quadratic contrasts of dArg:dLys ratio were also performed. Statistical significance was determined at $P \leq 0.05$. The local (pulp) and systemic (blood) inflammatory response to i.d. injected LPS was marked by a linear increase in heterophils with increasing dArg with a drop or no effect on lymphocytes, leading to changes in heterophil to lymphocyte ratios. On average, plasma AGP responded quadratically to dArg:dLys ratio while NO increased linearly at 6 h post-injection. Relative

mRNA expression of IL-1β, IL-6, and IL-8 in GF-pulps also responded in a dose-dependent manner. These data indicate that the GF model may be sensitive for detecting differences in immune responses of broilers fed different dietary treatments, and that Arg can influence the selected indicators of the LPS-induced inflammatory response in broilers.

INTRODUCTION

Arginine (**Arg**) is a dynamic amino acid (**AA**) that is nutritionally essential for poultry, as avian species are unable to synthesize Arg in sufficient quantities. Due to its involvement in the regulation of health and growth, Arg is also considered to be a functional AA as it has extensive secondary metabolic roles beyond protein synthesis and accretion, including a part in the regulation of the nonspecific innate immune system (Wu, 2009; Castro and Kim, 2020). Most likely, Arg influences innate immunity primarily through being the metabolic precursor for nitric oxide (**NO**), which acts not only as a toxic defense molecule against infectious organisms but also as a potent vasodilator and regulator of the functional activity, growth, and death of many cell types involved in immunity and inflammation including macrophages, T lymphocytes, antigen-presenting cells, mast cells, neutrophils, and natural killer cells (Coleman, 2001; Kröncke et al., 2001; Chen et al., 2008). During the immune response, NO is synthesized from L-Arg primarily by inducible nitric oxide synthase (**iNOS**) enzymes, which are regulated by cytokines and expressed widespread by activated immune cells following inflammation or infection (Green et al., 1994; Nathan and Xie, 1994a,b; Liew et al., 1997). Arginine may also enhance the total antioxidant capacity of the animal and alleviate oxidative stress by increasing the activity and expression of antioxidant enzyme families like glutathione peroxidase and superoxide dismutase (Duan et al., 2015; Liang et al., 2018) that can regulate inflammation.

In commercial broiler production, broiler health strongly depends on the defenses of the innate immune system given that their short production period is mainly during the developmental stage of their adaptive immunity (Hamal et al., 2006; Simon et al., 2014; French et al., 2020). Hence, potential optimization of the innate immune system responses using nutritional approaches like the manipulation of dietary Arg can be a natural and effective strategy for supporting broiler health, welfare, and production efficiency. The innate immune responses include the inflammatory response, which can be defined as a complex interplay of local and systemic changes that include the recruitment of leukocytes, plasma proteins, and fluids from the blood to infected tissue (French et al., 2020). In general, an inflammatory response that rapidly recruits a large number of the appropriate type of leukocytes, substantially alters the concentration of plasma acute-phase proteins, stimulates antimicrobial activities, and eliminates the original stimulating agent whereby a rapid return to biological homeostasis is achieved is desirable (Klasing, 1991; Juul-Madsen et al., 2014; Rocchi et al., 2023). Further research is needed to assess the dietary requirements of Arg that will optimize these immune functions for poultry, as they may not be the same as those required to optimize conventional targets such as growth performance and meat yield (Kidd, 2004).

Recently, French et al. (2020) reported on a "two-window" growing feather (**GF**) model used to evaluate the acute inflammatory responses of the broiler chicken innate immune system. This precision model induces the inflammatory response via intradermal (**i.d.**) injections of lipopolysaccharide (**LPS**) into GF-pulps. The GF-pulps are a skin derivative established as an accessible and minimally invasive tissue test site that allows for repeated sampling and monitoring of tissue responses to injected test material within an individual (Erf and Ramachandran, 2016). Multiple GF-pulps are injected with LPS at one time; then, at several time

points after injection paired samples of blood and GF tissue are collected simultaneously to provide the two-window insight into the development and resolution of the local and systemic inflammatory response. The objective of this current study was to utilize this established model to closely characterize the functional demands of dietary Arg and define its requirements during the inflammatory response of the broiler chicken innate immune system.

MATERIALS AND METHODS

The protocol for this study was reviewed and approved by the Division of Agriculture Institutional Animal Care and Use Committee at the University of Arkansas (animal use protocol #22047).

Bird Husbandry

Day-of-hatch male by-product breeder chicks from a Cobb 500 female line were obtained from Cobb-Vantress (Siloam Springs, AR, USA). A total of 490 broiler chicks that received a half-dose of Vaxxitek® HVT + IBD (Boehringer Ingelheim, Duluth, GA, USA) were neck-tagged upon arrival for individual identification and weighed by group to determine the overall average BW. Chicks were distributed to 70 battery cages (Alternative Design, Siloam Springs, AR, USA) with 7 birds per cage such that all group weights were within 2% of the overall expected group weight. At 14 d, bird number was reduced to 4 birds per cage and birds were reallotted to cages based on BW to reduce variability. Cages measured 0.61 × 0.61 m (0.37 m^2) to provide 0.05 m^2/bird for 7 chicks until 14 d of age and 0.09 m^2/bird for 4 birds until 42 d of age. Cages were equipped with a trough feeder and two drinker nipples to provide ad libitum access to feed and clean water throughout the trial. Supplemental chick feeders were placed in each cage from 0 to 7 d post-hatch to facilitate access to feed for young chicks. Birds were

monitored twice daily for any signs of distress (lethargy, impaired appetite, dehydration, etc.) and any birds that overtly exhibited these signs were euthanized using CO_2 inhalation or cervical dislocation. Light was continuously provided for the first 24 h and for 23L:1D during 1 to 4 d. Starting at 5 d, the light period and intensity were decreased appropriately until 29 d, at which point 16L:8D was sustained until the end of the trial. Brooding temperature was set to 32°C and decreased gradually throughout the trial to maintain bird comfort.

Dietary Treatments

Broilers were fed diets in three phases that consisted of a common starter diet (0 to 14 d) and experimental grower (14 to 28 d) and finisher (28 to 42 d) diets. Starter feed was crumbled while grower and finisher feeds were pelleted. Prior to formulation, main ingredients were analyzed for total AA profile and CP content by wet chemistry analysis to facilitate accurate diet formulation. The common starter diet was corn and soybean meal-based and formulated to meet or exceed the primary breeder nutrient recommendations (Cobb-Vantress, 2022), while the experimental grower and finisher diets were formulated to be nutritionally complete and meet industry specifications except for Arg concentration. For both experimental diet phases, the basal diet was corn and soybean meal-based with inclusion of distiller's dried grains with solubles, corn gluten meal, and wheat middlings in order to achieve the lowest target Arg ratio. Crystalline L-Arg (CJ Bio, Seoul, Korea) was then added to the basal diet at the expense of an inert filler to achieve five dietary digestible Arg:Lys ratios of 0.77, 0.91, 1.05, 1.19, and 1.33 (Table 3.1). These ratios ranged from below to well above the primary breeder recommendation for Arg during the experimental ages. Complete feeds from each growth phase were sampled and analyzed for nitrogen to determine CP via combustion (Method 990.03; AOAC International, 2007), total AA concentrations (Method 982.30 E [a, b, and c]; AOAC International, 2007), as

well as free Arg concentration (Method 999.13; AOAC International, 2007) at the Agricultural Experiment Station Chemical Laboratories at the University of Missouri-Columbia.

Growth Performance

Body weights and feed consumption were measured by pen at 0, 14, 28, and 35 d post-hatch to determine BW, body weight gain (**BWG**), feed intake (**FI**), and feed conversion ratio (**FCR**) for each feeding phase as well as cumulatively from 14 to 35 d. Feed intake was calculated based on bird days and FCR was corrected for mortality. Evaluation of growth performance was terminated at 35 d due to the intensiveness of the GF model sampling protocol. As such, a subset of 8 randomly selected birds per treatment (40 birds total) were chosen for use in the GF model over 39 to 42 d.

Induction of a Local Inflammatory Response in GF-Pulp by i.d. Injection of Lipopolysaccharide

At 20 d post-hatch, a row of 16 growing feathers on the breast feather tracts of each bird were plucked and allowed to regenerate for 19 d to yield rows of uniform GF for injection. At 39 d, 4 GF were collected from each bird as a pre-injection sample. At 40 d, 12 GF (6 per breast tract) from 6 birds per treatment were i.d. injected with 10 µL of *Salmonella* Typhimurium LPS (Sigma Chemical Company, Saint Louis, MO, USA) prepared in sterile, endotoxin-free Dulbecco's phosphate buffered saline (**PBS**; Sigma Chemical Company, Saint Louis, MO, USA) using 0.3 mL insulin syringes with 31 gauge × 8 mm needles, as previously described (Erf and Ramachandran, 2016; Sullivan and Erf, 2017). The LPS was diluted to a concentration of 100 µg/mL of PBS, providing 1 µg LPS per GF and 12 µg LPS per broiler. This dose has been established to reliably result in a local inflammatory response accompanied by detectable

inflammation-associated changes in the blood in modern broiler genotypes (French et al., 2020; Rocchi et al., 2023).

To determine local leukocyte infiltration as a response to the injection, 12 GF (6 per breast tract) from an additional 2 birds per treatment were i.d. injected with 10 µL of the PBS vehicle. The number of control birds available per treatment was limited due to the intensiveness of the sampling and processing protocols.

Blood and GF Sample Collections

Approximately 1.5 mL of blood and 4 GF were collected from every bird at each timepoint before (0 h) and at 6, 24, and 48 h post-i.d. GF-pulp injection. Blood was collected using 3 mL syringes with 25 gauge × 1 in needles, alternating between the left and right brachial vein at each timepoint and immediately transferred to 2 mL BD Vacutainer™ plastic tubes spray-coated with K_2EDTA as an anticoagulant. Blood samples were used to prepare diluted whole blood cell suspensions for immunofluorescence-based flow cytometry and for plasma analyses. To isolate plasma, EDTA blood was centrifuged at 400 × *g* at 4°C for 10 min. Plasma was then aliquoted and stored at -80°C for later analyses.

To collect GF, the GF was gently pulled in the opposite direction of feather growth while applying pressure to the skin. Of the 4 GF collected per time point, 2 GF were placed into a tube of ice-cold PBS and kept on ice until their preparation into pulp cell suspensions for various same-day laboratory analyses. The remaining GF were wrapped in aluminum foil, immediately flash-frozen in liquid nitrogen, and stored at -80°C for later RNA isolation.

Preparation of Whole Blood and GF-Pulp Cell Suspensions

Whole blood was diluted 1:50 by adding 20 µL of EDTA blood to 980 µL of PBS+ staining buffer (PBS, 1.0% bovine serum albumin, 0.1% sodium azide). The GF-pulp cell

suspensions were prepared as previously described (Erf and Ramachandran, 2016). Briefly, the feather sheath was cut longitudinally, and curved forceps were used to extract the entire pulp from both GF. The pulps were placed into 1.0 mL of PBS containing 0.1% collagenase type IV (Life Technologies, Carlsbad, CA, USA) and 0.1% dispase II (Boehringer Mannheim, Mannheim, Germany), and incubated in a 37°C water bath for 15 min. After incubation, the pulps were gently pushed through a 60 μm nylon mesh while being continuously flushed with ice-cold PBS. The pulp cell suspensions were washed twice in PBS at 250 × *g* at 4°C for 8 min and the final pellet was resuspended to a total volume of 0.35 mL using cold PBS. Cell suspensions were kept on ice until use for immunofluorescent staining.

Direct Immunofluorescent Staining of Blood and GF-Pulp Cell Populations and Analyses by Flow Cytometry

Populations of leukocytes in blood and GF-pulp cell suspensions were identified using direct, two- and three-color immunofluorescent staining procedures as previously described (Erf and Ramachandran, 2016; Rocchi et al., 2023) using several cocktails of fluorescently labeled mouse monoclonal antibodies (**mAb**) specific to chicken leukocyte markers. All mAb used were purchased from Southern Biotech (Birmingham, AL, USA) unless otherwise indicated. For blood cell suspensions, the first cocktail included mAb specific to chicken CD41/61 conjugated to fluorescein isothiocyanate (**FITC**; Bio-Rad Laboratories, Inc., Hercules, CA, USA) and CD45 conjugated to spectral red fluorochrome (**SPRD**) to identify thrombocytes and total leukocytes, respectively. The second mAb cocktail consisted of CD4-FITC (T helper cells), γδTCR conjugated to phycoerythrin (**PE**; γδ T cells), and CD8α-SPRD (cytotoxic lymphocytes), while the third consisted of KUL01-FITC (monocytes/macrophages), Bu-1-PE (B cells), and CD3-SPRD (T cells). The final whole blood dilution after immunofluorescent staining was 1:400. For

GF-pulp cell suspensions, the three mAb cocktails consisted of KUL01-PE and CD45-SPRD; gdTCR-FITC, CD4-PE, and CD8a-SPRD; and αβ1& αβ2 TCR-FITC (αβ T cells), Bu-1-PE, and CD45-SPRD. The isotype of the mouse antibodies used for cell suspensions was IgG1. Appropriate controls were also included in the analyses to determine non-specific binding of directly labeled mouse IgG1 antibodies, to establish the threshold between negative and positive fluorescence, and to set compensations (French et al., 2023). Because antibodies specific for chicken heterophils are not available, heterophil populations were estimated based on the size (forward scatter) and internal complexity/granularity (side scatter) of blood ($CD45^+$ $CD41/61^-$) and pulp ($CD45^+$) leukocytes. All cell samples were analyzed by flow cytometry using a BD Accuri C6-plus flow cytometer (Becton Dickinson, San Jose, CA, USA) and FlowJo software (Ashland, OR, USA). Blood cell data were expressed as concentrations (cells/μL), whereas pulp data were expressed as the percentage of cells in the total pulp cell suspension (% pulp cells) to allow for comparison of samples on a relative quantitative basis. Total lymphocytes were calculated as the sum of T and B cells, and heterophil to lymphocyte (**H:L**) ratios were calculated by dividing the estimates of heterophils by that of total lymphocytes for GF-pulp and blood, respectively.

RNA Isolation and Relative Gene Expression Analyses of Cytokines from GF-pulps by qPCR

Total RNA was isolated from 2 frozen GF-pulps pooled for each bird at each time point using the Zymo Research Direct-zol RNA Miniprep kit (Zymo Research, Irvine, CA, USA) as described by French et al. (2020). The concentration and purity of isolated RNA was assessed by NanoDrop (Thermo Fisher Scientific, Waltham, MA, USA). Total RNA was reverse-transcribed to cDNA using ABScript III RT Master Mix for qPCR (ABclonal Technology, Woburn, Massachusetts, USA) following the manufacturer's protocol. Synthesized cDNA was diluted to

10 ng/μL using nuclease-free water and stored at -20°C until further processing for relative gene expression analysis.

For relative gene expression analysis of cytokines, primer-probe assays for interleukin 1β (IL-1β), IL-6, and IL-8/CXCL8 and the 28S housekeeping gene were used for qPCR procedures as described by French et al. (2020) (Supplemental Table 3.1). Real-time qPCR was performed in the 7500 Fast Real-Time PCR system (Thermo Fisher Scientific) using Entrans 2X qPCR Probe Master Mix (Abclonal Technology). For each target gene expressed in a sample, delta Ct (ΔCt) was calculated by subtracting the Ct value of the 28S housekeeping gene from that of the target gene Ct value, and relative gene expression was calculated as 40 minus ΔCt (Rocchi et al., 2023).

Plasma Nitric Oxide and Alpha-1-Acid Glycoprotein Analyses

Nitric oxide concentration (μM) in the plasma of blood samples collected before (0 h), and 6, 24, and 48 h post i.d. GF-pulp injection of LPS or PBS was determined by colorimetric assay following the manufacturer's protocol (Cayman Chemical Company, Ann Arbor, MI). The sum of both nitrate (NO_3^-) and nitrite (NO_2^-) were used to determine total NO. Prior to analysis, plasma samples were deproteinized using pre-rinsed Amicon® Ultra 30,000 MWCO filters (Millipore Sigma, Burlington, MA) to remove interfering proteins with a molecular weight greater than 30 kilodaltons. Plasma samples were diluted 1:2 with assay buffer prior to beginning the assay. Alpha-1-acid glycoprotein concentration (mg/mL) in the plasma of the same blood samples was determined by chicken AGP ELISA according to instructions provided by the manufacturer (Life Diagnostics, West Chester, PA). Plasma samples were diluted 1:20,000 with assay diluent prior to conducting the assay.

Statistical Analyses

Growth performance data were analyzed by one-way ANOVA for a randomized complete block design with cage as the experimental unit and cage location as a random blocking factor. All data from the GF model were analyzed by three-way ANOVA to determine the effects of injection (LPS, PBS), time (0, 6, 24, 48 h), and diet (0.77, 0.91, 1.05, 1.19, and 1.33 dArg:dLys ratio), and their interactions on each dependent variable. As there were significant interactions involving injection type, data for LPS and PBS injections were separately analyzed by two-way repeated measures ANOVA for blood or two-way ANOVA for GF data, with time and diet as main effects. In a case where the Shapiro-Wilk normality test failed ($P > 0.05$), log base 10 transformations were utilized to restore normality assumptions. Linear and quadratic contrasts of dArg:dLys ratio were also performed to examine the effects of increasing dietary Arg concentration on each dependent variable. In all cases, statistical significance was determined at $P \leq 0.05$, and all data were analyzed using JMP Pro 17.2 software (SAS Institute, Cary, NC).

RESULTS

Diet Analyses

Calculated and analyzed values for the common starter diet as well as the experimental grower and finisher diets are shown in Table 3.1. As anticipated, analyzed total and free Arg concentration increased proportionally as calculated digestible Arg values increased. Free Arg concentration in the 0.77 dArg:dLys diet was 0.3% for both grower and finisher phases, and this value increased on average by 0.11% for each subsequent diet up to 0.46% for 1.33 dArg:dLys.

Growth Performance

Average BW of each dietary treatment was not different ($P > 0.05$) prior to the start of the cumulative experimental growth period. By the end of the grower period, there was a linear increase in BW ($P < 0.0001$) and BWG ($P < 0.0001$) as dArg:dLys increased, while FCR quadratically decreased ($P = 0.001$) (Table 3.2). These effects persisted through the cumulative 14 to 35 d post-hatch period, for BW ($P = 0.0001$), BWG ($P = 0.0001$), and FCR ($P < 0.0001$). Increasing dArg:dLys increased cumulative BWG by 147 g from 2.126 kg at 0.77 dArg:dLys to 2.273 kg at 1.33 dArg:dLys, and FCR was highest at 0.77 dArg:dLys (1.666) but improved quadratically 13 points to 1.19 dArg:dLys.

GF-Pulp and Blood Leukocyte Population Profiles

Heterophils. There were no differences ($P > 0.05$) in the estimated levels of heterophils in the GF-pulps or the peripheral blood due to dArg:dLys ratio prior to i.d. GF-pulp injection with LPS. For LPS injected broilers, proportions (% pulp cells) of heterophils in the pulp increased at 6 h p.i., then dropped by 24 and 48 h, but remained above pre-injection levels (Figure 3.1A). There was no dArg:dLys by time interaction, and there was a significant ($P < 0.0001$) main effect of time. Furthermore, a linear contrast of the main effect of dArg:dLys ($P = 0.022$) showed that heterophil proportions in GF-pulps increased as dArg:dLys increased (Figure 3.1C). On average, heterophil proportions ranged from 6.51% at 0.77 dArg:dLys to 10.14% at 1.33 dArg:dLys. This positive linear response was most influenced by values at 6 h p.i., at which individual time point heterophil proportions also linearly increased with dArg:dLys ($P = 0.023$; contrast not shown).

In the blood, heterophil concentrations (cells/μL) were also elevated at 6 h p.i. but returned to baseline levels by 24 to 48 h (Figure 3.1B). Similar to the response in GF-pulps, there

was no dArg:dLys interaction, a significant ($P < 0.0001$) main effect of time, and heterophil concentrations linearly increased ($P = 0.001$) with higher dArg:dLys (Figure 3.1C).

In the GF-pulps of PBS injection control birds, there was also a minor elevation in the percentage of heterophils in the tissue at 6 h p.i. that resulted in a significant effect of time ($P = 0.001$; Supplemental Figure 3.1). However, this increase in heterophils for PBS controls was 3-fold relative to pre-injection levels versus 28-fold following LPS and was likely due to slight injury of the tissue from injection. Heterophil proportions returned to pre-injection levels by 24 to 48 h, and there was no effect of dArg:dLys on their recruitment to GF tissue. Heterophil concentrations in the blood did not change in response to i.d. GF-pulp injection of PBS either due to time ($P = 0.476$) or dArg:dLys ($P = 0.662$).

Lymphocytes. There were no differences ($P > 0.05$) in lymphocyte levels (total lymphocytes or separate T and B cells) in the GF-pulp or the peripheral blood due to dArg:dLys ratio prior to i.d. GF-pulp injections. For LPS injected broilers, proportions (% of pulp cells) of lymphocytes in the pulp showed no dArg:dLys by time interaction after injection and were not affected by time ($P = 0.292$; Figure 3.1D); however, lymphocyte levels linearly ($P < 0.0001$) decreased as dArg:dLys increased (Figure 3.1F). This linear decrease in total lymphocytes was due to both T cells ($P = 0.003$; Figure 3.2A and 3.2C) and B cells ($P = 0.005$; Figure 3.2D and 3.2F). Total lymphocyte proportions in GF-pulp averaged across time points decreased from 2.59% at 0.77 dArg:dLys to 1.10% at 1.33 dArg:dLys.

In the blood, concentrations of total lymphocytes decreased on average by more than 2-fold at 6 h p.i., from 22.31×10^3 to 9.63×10^3 cells/μL, with T cells returning to pre-injection levels by 24 h and B cells remaining numerically below (Figure 3.1E; Figure 3.2B and 3.2E) throughout the 48-h examination period. There were no dArg:dLys by time interactions, but the

decrease in lymphocyte concentrations at 6 h p.i. resulted in a significant main effect of time for total lymphocytes ($P < 0.0001$), individual T cells ($P < 0.0001$), and B cells ($P < 0.0001$). Dietary dArg:dLys had no effect on the concentrations of lymphocytes in the blood (Figure 3.1F; Figure 3.2C and 3.2F).

Injection of PBS (injection control) into GF-pulps did not result in any significant lymphocyte level changes. However, in the blood, PBS injection resulted in a significant main effect of time ($P = 0.002$), where total lymphocyte concentrations (mainly due to B cells) decreased just over 1-fold at 6 h p.i. before returning to baseline (0 h) levels (Supplemental Figures 3.1 and 3.2). As discussed, this drop at 6 h was also observed post-LPS injection, but with over twice the magnitude.

Heterophil to Lymphocyte Ratios. Before i.d. GF-pulp injections, there were no differences ($P > 0.05$) in the ratio of heterophils to total lymphocytes in either the GF-pulp or peripheral blood due to dArg:dLys ratio. For LPS injected broilers, there were no dArg:dLys by time interactions but a significant main effect of both time ($P < 0.0001$) and dArg:dLys ($P = 0.002$) on H:L in GF-pulps (Figure 3.1G). The highest H:L ratios were observed at 6 h p.i., but approached baseline values by 48 h. Furthermore, with increasing dArg:dLys ratio the H:L linearly increased ($P = 0.0001$; Figure 3.1I).

In the blood, H:L concentration ratios mirrored the same statistical effects as in GF-pulp (Figure 3.1H). However, in contrast to GF-pulp, the increase in H:L ratio at 6 h p.i. was caused by both an increase in heterophils and a drop in lymphocytes (Figure 3.1I; Figure 3.1B and 3.1E).

There were no significant changes in H:L in either GF-pulps or blood as a result of i.d. GF-pulp injection of PBS (injection control; Supplemental Figure 3.1).

Macrophages/Monocytes. Prior to i.d. GF-pulp injection, there were no differences ($P > 0.05$) in either the proportions of macrophages in GF-pulps or concentration of monocytes in the peripheral blood due to dArg:dLys ratio. In LPS injected broilers, there were no dArg:dLys by time interactions for either GF-pulp macrophages or blood monocytes, and macrophage levels in the pulp increased over time ($P < 0.0001$), independent of dArg:dLys (Figure 3.2G). Monocyte concentrations responded quadratically over time ($P < 0.0001$) by increasing at 6 h p.i. and returning to pre-injection levels by 24 h (Figure 3.2H). Additionally, a linear contrast ($P = 0.053$; Figure 3.2I) showed a tendency for monocyte concentrations to increase with increasing dArg:dLys.

Growing feather-pulp injection of PBS (injection control) did not result in any macrophage or monocyte changes in the pulp or peripheral blood, respectively (Supplemental Figure 3.2).

Plasma Nitric Oxide Concentration

Prior to i.d. GF-pulp injection, plasma concentrations (μM) of NO were not different ($P > 0.05$) due to dArg:dLys ratio (Figure 3.3). For LPS injected broilers, there was a significant dArg:dLys by time interaction that resulted primarily from the concentrations observed at 6 h p.i. versus other time points. Notably, NO in the plasma at 6 h increased linearly as dArg:dLys increased ($P < 0.0001$), but no other consistent trends regarding NO concentration and dArg:dLys ratio at 0, 24, or 48 h p.i. were observed. Moreover, the i.d. LPS injection also resulted in the greatest average plasma NO concentration at 6 h p.i., regardless of dArg:dLys, whereby NO increased 7-fold from 11.47 μM at 0 h to 79.96 μM at 6 h p.i, returning to baseline levels by 24 h (10.76 μM).

Growing feather-pulp injection of PBS (injection control) did not result in any meaningful effects on plasma NO due to time post-injection or dArg:dLys.

Plasma Alpha-1-Acid Glycoprotein Concentration

Prior to i.d. GF-pulp injections, there were no differences ($P > 0.05$) in the plasma concentrations (mg/mL) of AGP due to dArg:dLys ratio (Figure 3.4). For LPS injected broilers, there was no dArg:dLys by time interaction and there was a significant main effect of time ($P < 0.0001$), whereby AGP concentration at 0 h increased 3-fold to 2.51 mg/mL by 24 h p.i. before beginning to decrease by 48 h. There was also a significant quadratic effect of dArg:dLys ($P < 0.0001$), where the lowest average AGP concentration of 0.99 mg/mL was observed at 0.77 dArg:dLys and the highest of 1.97 mg/mL at 1.19 dArg:dLys, before concentration began to decrease at 1.33 dArg:dLys (1.61 mg/mL).

In PBS injection control birds, there were no significant changes in plasma AGP concentration as a result of i.d. GF-pulp injections.

Relative Cytokine mRNA Expression in GF-Pulps

Prior to i.d. GF-pulp injections, relative mRNA expression levels of IL-1β and IL-8 were not different due to dArg:dLys ratio ($P > 0.05$; Figure 3.5). However, expression of IL-6 was significantly lower in the pulps of broilers fed the two lowest dArg:dLys compared with the pulps of those fed higher ratios. For LPS injected broilers, there were no significant dArg:dLys by time interactions but there were significant main effects of both time and dArg:dLys on relative expression of each measured cytokine. The main effect of time resulted from an increase in relative expression of all cytokines at 6 h p.i. that either numerically decreased (IL-1β, IL-6) or continued to increase (IL-8) thereafter. For IL-1β and IL-8, increasing dArg:dLys linearly increased ($P = 0.007$ and 0.002, respectively) relative expression, whereas IL-6 also increased

quadratically ($P = 0.017$) and had the numerically highest relative expression at 1.19 dArg:dLys. Though there were no significant dArg:dLys by time interactions, all significant main effects of dArg:dLys were mainly driven by the separation of dietary responses at 24 and 48 h p.i., as relative mRNA expression was generally similar for each cytokine at 0 and 6 h p.i. The pre-injection differences in expression of IL-6 mRNA in GF-pulps were no longer observed post-LPS injection ($P > 0.05$).

In PBS injection control birds, there were no significant changes in the relative expression of IL-1β mRNA as a result of i.d. GF-pulp injections. For IL-6 and IL-8, two-way ANOVA and orthogonal polynomial contrast *p*-values were not estimable due to an insufficient amount of cDNA from some dArg:dLys for analysis. However, on average the relative expressions of these inflammatory cytokines were lower for PBS-injected birds than they were for LPS-injected birds.

DISCUSSION

Growth Performance. Results from growth performance measurements of this trial show that BW, BWG, and FCR are responsive to changes in dArg:dLys ratio over the range of 0.77 to 1.33. These results also support evidence that Arg was the limiting nutrient in the experimental diets.

Leukocyte Profiles. The leukocyte population profiles measured in the GF-pulp and blood at the individual time points in this study were typical of the broiler inflammatory response caused by i.d. injection of the selected dose of LPS from *Salmonella* Typhimurium as characterized by previous studies (French et al., 2020; Rocchi et al., 2023). More importantly, these responses also reflect the patterns of action generally expected of the acute phase

inflammatory response to LPS in the chicken (Byrne and Erf, 2024). Heterophils are typically the first and most abundant leukocytes to infiltrate the site of LPS-injection, reaching peak levels within h and returning to baseline levels 24 to 48 h later (Maxwell and Robertson, 1998; More Bayona et al., 2017; Byrne and Erf, 2024). In our study, we observed a rapid increase of heterophils in both the GF-pulp and blood at 6 h p.i. that mostly subsided by 48 h. Along with heterophils, monocytes are recruited from the blood to the affected tissue albeit less rapidly (shown in our data at 6 h p.i.; Chansoriya et al., 1993; French et al., 2020). Once in the tissue, monocytes differentiate into macrophages (French et al., 2020), capable of producing pro-inflammatory cytokines, and restoring homeostasis and wound healing during the later phase of the acute inflammatory response which likely explains the linear increase in GF-pulp proportions over time. A sizeable decrease in total as well as individual T and B cells in the blood at 6 h p.i. also occurred. This response has also been observed in other studies using various types of LPS administration in chickens and has been attributed to toxic effects of LPS on lymphocytes, the redistribution of lymphocytes from the blood to organs, and/or physiological stress effects associated with GF-pulp injection and handling of the birds (Shini et al., 2008; Bowen et al., 2009; French et al., 2020; Rocchi et al., 2023; Byrne and Erf, 2024).

In our study, the dietary dArg:dLys ratio had significant effects on the proportions and concentrations of various leukocytes in the GF-pulp and blood, respectively. As mentioned, increased dArg:dLys linearly increased heterophil populations in the GF-pulp and blood, linearly decreased the proportions of total as well as individual T and B lymphocytes in the GF-pulp and linearly increased the concentration of monocytes in the blood. The mechanisms by which dietary Arg can affect leukocyte populations require further investigation. In fact, few studies have researched the effect of dietary Arg on leukocytes in general during the acute phase of the

inflammatory response, and most evaluate percentage data making it difficult to compare directly to blood concentration data as presented in our study. Lee et al. (2002) reported that both the percentage and absolute number of heterophils increased linearly in the blood of birds fed increasing concentrations of dietary Arg 1 d after an infectious bronchitis challenge, but not at 7 d. The mechanism for this was not evaluated, but other studies have shown that NO can increase migration of mouse neutrophils (the mammalian counterpart to avian heterophils) to inflammatory sites (Ajuebor et al., 1998; Cuzzocrea et al., 2000; Franco-Penteado et al., 2001). It is also interesting to note that the cationic antimicrobial peptides contained in the granules of heterophils are rich in Arg (Harmon et al., 1992; Mukhopadhyay et al., 2010). However, there is no evidence to suggest that increased availability of Arg for synthesis of these peptides would significantly alter concentrations of heterophils in blood or tissue.

Other studies have also shown that dietary Arg can influence T and B lymphocyte proportions in chickens, but the responses reported vary. Abdukalykova et al. (2008) measured a higher percentage of $CD3^+$ (T cells), Bu-1^+ (B cells), and $CD4^+$ (helper T) cells in the blood of broilers fed 2.2% Arg diets compared to those fed 1.2% Arg after vaccination for infectious bursal disease, and Lee et al. (2002) reported that the percentage of $CD8^+$ (cytotoxic T) cells in the blood of birds fed adequate dietary Arg was greater than those fed deficient diets 7 d post infectious bronchitis vaccine. D'Amato and Humphrey (2010) showed that the percentage of B cells in the blood of young broilers tended to increase with increasing Arg in unchallenged birds, but Tan et al. (2014a) reported a decrease in the percentage of peripheral blood B cells as well as splenic lymphocytes in broilers fed increasing Arg (up to 2.53% total Arg) after LPS challenge. The latter result was suggested to be from an increase in NO-mediated macrophage production of highly reactive oxidants. In our study, the decrease in GF-pulp (tissue) lymphocytes was reported

within 48 h of the i.d. GF-pulp injection of LPS, which is a time point earlier than measured in the aforementioned studies and not likely reflective of adaptive immunity. Although it is possible that high concentrations of NO associated with higher dArg:dLys inhibited T cell proliferation or caused apoptosis of lymphocytes (Coleman, 2001) in the GF tissue, the decrease in their proportions with increased dArg:dLys was likely a result of relatively higher recruitment of heterophils to the inflammatory site (GF-pulp) rather than a decrease in lymphocyte concentration per se.

The influence of dietary Arg on chicken blood monocyte concentrations reported in the literature also varies. D'Amato and Humphrey (2010) found that broilers fed 1.5% total dietary Arg had a higher percentage of monocytes in peripheral blood at 14 d compared to those fed 1.1% or 1.3% total Arg, and Cengizi and Küçükersan (2010) reported an increase in monocyte concentration in 42 d broilers fed diets supplemented with up to double the NRC (1994) recommendation for Arg. However, Lee et al. (2002) found no effect of dietary Arg level on the concentration of circulating monocytes in egg-type chickens at either 1 or 7 d after vaccine, and Kidd et al. (2001) showed no change in the monocyte percentage of healthy broilers fed supplemental Arg compared to a control diet. Inconsistencies between studies may be due to differences in the way data are expressed (percentage versus concentration), and also variations in environmental conditions, dietary Arg concentrations, bird genetics, sampling time points, and other experimental factors.

Heterophil to Lymphocyte Ratio. The blood H:L ratio is a widely used indicator of the physiological stress response in birds. During mild or moderate stress conditions in healthy individuals, blood heterophil numbers increase, and the H:L ratio can therefore be used to detect the presence of such physiological states (Maxwell and Robertson, 1998). In our study, the

increased H:L ratios in the GF-pulps and blood following LPS injection were mainly due to the LPS-induced heterophil migration to the injected GF-pulp and signals to the bone marrow to produce and release heterophils into the blood, respectively. The heterophil response was greatest at 6 h p.i., where differences among dietary treatments contributed most to the overall linear increase in H:L ratio observed with increasing dArg:dLys ratio. Traditionally, a higher H:L ratio is associated with a greater level of physiological stress, and Gross and Siegel (1983) reported that blood H:L values of 0.20 and 0.80 are characteristic of low and high degrees of stress, respectively. On average, 0.77 and 1.33 dArg:dLys in our study resulted in a blood H:L of 0.42 and 1.08, respectively. However, it is important to note that in our experiment the physiological stress was experimentally induced by the i.d. GF-pulp injection of LPS, and that in general, an inflammatory response that works rapidly to restore homeostasis is desirable (Rocchi et al., 2023). Thus, in this case, a higher H:L ratio in response to increasing dietary Arg could indicate an enhanced capacity of the immune system to respond to inflammatory agents. Indeed, Lee et al. (2002) reported an increase in H:L ratio with higher concentrations of Arg 1 d after an infectious bronchitis vaccine was administered to egg-type chickens and suggested that this may be indicative of an improved phagocytic ability of the immune system during early stages of a viral infection. Alternatively, Lieboldt et al. (2017) found no differences in H:L up to 48 h after laying-breed cockerels were intramuscularly injected with LPS and fed increasing concentrations of dietary Arg ranging from 70 to 200% of the NRC (1994) recommendation. In the absence of an immune challenge, Al-Daraji and Salih (2012) showed that supplemental Arg in broiler diets (up to 0.06%) significantly decreased H:L ratios, and that the highest Arg concentration was associated with the lowest H:L.

Additional Systemic Responses to GF-Pulp Injection of LPS

Nitric Oxide. After i.d. GF-pulp injection with LPS, NO concentration in the plasma increased 7-fold irrespective of diet from 0 to 6 h p.i, returning to baseline levels by 24 h. Nitric oxide is a highly reactive biological nitrogen free radical that acts as a toxic defense molecule and as a complex regulator of immune and inflammatory function through multiple pathways (Fang and Li, 1989; Wink et al., 1996; Guo et al., 2015). It is released by activated leukocytes in response to inflammatory stimuli such as bacterial LPS (Takahashi et al., 1999; Chapman and Wideman, 2006), and thus, as expected in the current study, plasma concentrations of NO markedly increased following i.d. GF-pulp injection of LPS. At high NO concentrations, such as those observed at 6 h p.i. in our study, the effect of the molecule on immunity is mainly indirect (Coleman, 2001). High concentrations of NO (in the micromolar range) are most likely of iNOS origin and can cause the production of reactive nitrogen oxide species that induce cell toxicity and lipid peroxidation, inhibit mitochondrial respiration, and either enhance or inhibit a range of key signaling proteins and enzymes like members of the MAP kinase family (Stamler, 1994; Wink et al., 1996; Coleman, 2001; Schindler and Bogdan, 2001; Hirst and Robson, 2011). Nitric oxide also exerts effects at lower concentrations, though, and in fact, differences in levels of NO that are biologically effective may be small enough to go undetected by commercial assays (Chapman and Wideman, 2006). Additional NO effects on immunity include its action as a vascular relaxing agent aiding leukocyte recruitment and extravasation, an inhibitor of platelet aggregation, and either an inducer or suppresser of cellular apoptosis (Coleman, 2001; Kröncke et al., 2001; Chapman and Wideman, 2006; Hirst and Robson, 2011). It is possible that these functions are also due to constitutively expressed isoforms of NOS, despite typical production of

NO at smaller concentrations and for shorter durations than the inducible isoform (Coleman, 2001; Schindler and Bogdan, 2001).

Because NO has significant roles in the regulation of immunity and other biological processes, it is often recognized to be the primary mediator of the immunomodulatory effect of Arg (Hassan et al., 2021). In our study, not only did 6 h post i.d. GF-pulp injection with LPS result in the greatest overall production of NO, but it also resulted in a linear increase in NO production at this individual time point as dArg:dLys increased. As Arg is the only known biological precursor for production of NO (Coleman, 2001; Fernandes and Murakami, 2010), the linear as opposed to quadratic increase in concentration in our study shows that even at a dArg:dLys of 1.33, Arg was likely still a limiting nutrient for NO production. Other studies have also reported that NO concentration increases with dietary Arg concentration (Khajali et al., 2011; Basoo et al., 2012; Guo et al., 2015), and results from Takahashi (1999) show that NO production can be proportionally affected by Arg concentration or intake. In this study, broilers were fed diets deficient (0.65%), sufficient (1.44%), and in excess (2.44%) of dietary Arg before abdominal injection with LPS, which resulted in a plasma nitrite concentration that increased with dietary Arg during peak NO concentrations (6 and 10 h post-injection).

It should also be considered that the optimal level of NO production may not be the maximum amount, as high levels of NO can cause NO-mediated toxicity and cellular damage through formation of highly reactive oxidants (peroxynitrite anion; Wink et al., 1996; Coleman, 2001). However, Guo et al. (2015) showed that 21 d old broilers fed increasing concentrations of Arg experienced a quadratic increase in iNOS activity in the serum as well as iNOS expression in the liver and spleen, indicating that there is likely a threshold of Arg at which further iNOS expression will be suppressed.

Alpha-1-Acid Glycoprotein. After i.d. GF-pulp injection with LPS, AGP concentration in the plasma increased 3-fold irrespective of diet from 0 to 24 h p.i. before beginning to decrease. In chickens, AGP is a moderate positive acute phase protein that is synthesized mainly by hepatocytes in response to systemic tissue injury, inflammation, or infection, and acts as an important binding protein in plasma as well as an immunomodulatory agent with both pro- and anti-inflammatory properties (O'Reilly and Eckersall, 2014). Upon stimulation, the concentration of AGP in chicken serum can increase 10- to 100-fold during acute phase reactions, making it a useful biomarker for inflammation (Fournier et al., 2000; Gruys et al., 2005; Juul-Madsen et al., 2014). After injection with LPS, plasma AGP concentration of chickens generally peaks between 24 and 48 h, with higher concentrations of LPS resulting in higher concentrations of AGP (Nakamura et al., 1998; Takahashi et al., 1998; Adler et al., 2001; Rocchi et al., 2023). Alpha-1-acid glycoprotein may help with LPS clearance by binding to it directly, neutralizing its toxicity (Moore et al., 1997; Juul-Madsen et al., 2014).

In our study, there was an overall quadratic increase in AGP concentration irrespective of time point as dArg:dLys increased. Arginine may influence the plasma concentration of AGP either directly, potentially by being a limiting component in the synthesis of the glycoprotein itself, or indirectly, for example by its influence on more core regulators of the inflammatory response. Nitric oxide, for example, together with glucocorticoids and proinflammatory cytokines can cause changes in plasma concentrations of acute phase proteins following immunostimulation (Gruys et al., 2005). Moreover, a study by Itoh et al. (1994) reported that in vitro, AGP production from nonparenchymal rodent hepatic cells is primarily mediated by the NOS pathway, and possibly, by secreting NO. Thus, although not the main regulatory

mechanism, it is possible that L-Arg metabolism in the liver can modify AGP secretion in vivo through several mechanisms.

Additional Local (Tissue) Responses to GF-Pulp Injection of LPS

Relative mRNA Expression of Cytokines. After i.d. GF-pulp injection with LPS, relative mRNA expression of all inflammatory cytokines measured in this study were increased at 6 h p.i. By 24 and 48 h p.i., relative expression had either begun to decrease (IL-1β, IL-6), or continued to increase (IL-8). Lipopolysaccharide initiates the inflammatory response by binding to Toll-like receptor 4 (**TLR4**), which is expressed on the surface of leukocytes like monocytes/macrophages and heterophils (Dil and Qureshi, 2002; Kogut et al., 2005; Kannaki et al., 2010). After binding of LPS, macrophages produce and release vasoactive factors including proinflammatory cytokines (IL-1β, IL-6, TNF-α) and chemokines (IL-8) that lead to vasodilation, induction of fever, release of acute phase proteins, and chemotaxis of immune cells like heterophils to the site of inflammation (Remick, 2005; Xu et al., 2011; Tanaka et al., 2014; Galozzi et al., 2021). Thus, the upregulation of these inflammatory cytokine genes by LPS in our study, particularly at 6 h p.i., is in agreement with previous findings. Interleukin-1β is an especially potent proinflammatory mediator whose gene expression in GF-pulp has been shown to be greatly elevated between 4 and 8 h post-LPS injection in chickens, which decreases by 24 to 72 h despite remaining above pre-injection levels (French et al., 2020; Galozzi et al., 2021; Bryne and Erf, 2024). In these studies, expression of IL-8 mRNA responded similarly; however, Rocchi et al. (2023) showed that expression of IL-8 remained elevated at 24 h similar to that of 6 h p.i. in broilers. In humans, it has been reported that IL-8 expression can remain upregulated at sites of acute inflammation for several days post stimulation (Remick, 2005).

Relative expression of all inflammatory cytokines measured in this study were affected in some dose-dependent manner by increasing dietary dArg:dLys. Interleukin-1β and IL-8 showed a linear increase, while the increase in IL-6 expression was quadratic. Most likely, Arg influences inflammatory cytokine gene expression primarily through NO, as NO generated by macrophages can signal to lymphocytes and regulate the secretion of cytokines by nitrosylation of proteins in pathways like IκB/NFκB and JAK/STAT (Coleman, 2001; Schindler and Bogdan, 2001). In addition, high concentrations of NO during immune stimulation can induce T cells to secrete additional cytokines (Xu et al., 2011). Beyond direct NO mediation, Mieulet et al. (2010) showed that depletion of Arg inhibited proinflammatory cytokine production in LPS-stimulated mouse macrophages by impairing the activation of the ERK1/2 signaling cascade, correlating Arg deficient diets to decreased cytokine production. However, several studies suggest that increased dietary Arg may decrease inflammatory cytokines/gene expression in serum and various tissues such as spleen and intestine by reporting that Arg can attenuate inflammation in broilers through the potential suppression of the TLR4 pathway (Tan et al., 2014a,b; Zhang et al., 2019; Izadi Yazdanabadi et al., 2020; Zhang et al., 2020). Just as with H:L ratios, perhaps the increase in relative expression of inflammatory cytokines with increased dArg:dLys observed in our study is reflective of an enhanced capacity for the immune system to respond more acutely to inflammatory stimuli, altering the patterns of inflammation that may be observed longer-term. Ultimately, differences in cytokine responses to Arg among studies are likely due to the inflammatory models used and the time points selected for sampling (d versus h after immune stimulation).

Overall, the results of this study confirm not only that the “two-window” GF model can be sensitive for detecting differences in immunomodulatory responses of broilers fed different

dietary treatments, but also that dietary dArg:dLys ratio clearly influences selected indicators of the broiler chicken acute (0 to 48 h) inflammatory response after i.d. GF-pulp injection with LPS. Linear responses to increasing dArg:dLys ratio beyond the highest ratio of 1.33 indicate that Arg requirements to support these measured responses may be higher than those currently recommended to optimize growth performance and meat yield.

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Table 3.1. Ingredient, calculated, and analyzed nutrient composition of the common starter and experimental grower and finisher Diet A (% as-is, unless otherwise noted).

		Experimental[1]	
Item	Starter (0 to 14 d)	Grower (14 to 28 d)	Finisher (28 to 42 d)
Corn	58.00	65.41	66.79
Soybean meal	31.45	10.31	10.49
DDGS[2]	3.00	10.00	10.00
Corn gluten meal	3.00	5.00	5.00
Wheat middlings	-	4.00	2.75
Meat and bone meal	1.38	-	-
Poultry fat	0.50	1.10	0.79
Calcium carbonate	1.04	0.97	0.97
Dicalcium phosphate	0.50	0.62	0.63
Sodium bicarbonate	-	0.32	0.33
Salt	0.31	0.06	0.06
Inert filler (sand)	-	0.54	0.54
L-Lysine·HCL	0.28	0.55	0.55
L-Methionine	0.26	0.21	0.21
L-Threonine	0.08	0.19	0.18
L-Valine	-	0.14	0.14
L-Isoleucine	-	0.13	0.13
L-Tryptophan	-	0.05	0.05
Glycine	-	0.22	0.22
Choline chloride	0.05	-	-
Trace mineral premix[3]	0.10	0.10	0.10
Vitamin premix[4]	0.05	0.03	0.03
Xylanase[5]	-	0.03	0.03
Phytase[6]	0.01	0.01	0.01
Calculated composition			
ME, kcal/kg	3,051	3,153	3,153
CP	23.53	18.41	18.37
Digestible Lys	1.24	0.96	0.96
Digestible Arg	1.33	0.74	0.74
Total Ca	0.90	0.72	0.72
Available P	0.45	0.36	0.36
Analyzed nutrients[7,8]			
CP	22.49	19.01	18.53
Total Lys	1.24	1.07	1.12
Total Arg	1.23	0.89	0.90

[1]Crystalline L-Arginine was added to Diet A (calculated dArg:dLys ratio of 0.77) at the expense of inert filler in order to meet calculated dArg:dLys ratios for Diets B-E (0.91, 1.05, 1.19, and 1.33, respectively).
[2]Distillers dried grains with solubles.
[3]The mineral premix provided (per kg of diet): calcium, 81.0 mg; manganese, 100.0 mg; zinc, 100.0 mg; copper, 15.0 mg; iron, 15.0 mg, iodine, 1.5 mg; selenium, 0.25 mg.

[4]The vitamin premix provided (per g of premix): vitamin A, 30,863 IU; vitamin D_3, 22,045 ICU; vitamin E, 220 IU; vitamin B_{12}, 0.1 mg; menadione, 6.0 mg; riboflavin, 26.5 mg; d-pantothenic acid, 39.7 mg; niacin, 154.3 mg; folic acid, 3.5 mg, pyridoxine, 11.0 mg; thiamine, 6.2 mg; biotin, 0.3 mg.
[5]Hostazym X (Huvepharma, Sofia, Bulgaria).
[6]OptiPhos Plus® (Huvepharma, Sofia, Bulgaria).
[7]The analyzed total Arg:Lys ratios for experimental grower diets B-E were 0.85, 0.98, 1.09, and 1.16, respectively.
[8]The analyzed total Arg:Lys ratios for experimental finisher diets B-E were 0.88, 0.97, 1.11, and 1.21, respectively.

Table 3.2. Growth performance of Cobb 500 male broilers fed increasing concentrations of Arg from 14 to 35 d post-hatch[1].

	Arg Ratio						*P*-values		
Item	0.77	0.91	1.05	1.19	1.33	SEM	ANOVA	Linear	Quadratic
14 to 28 d – Grower Period									
14 d BW, kg	0.582	0.581	0.585	0.582	0.584	0.002	0.616	0.301	0.845
28 d BW, kg	1.880^{c}	1.940^{bc}	1.981^{ab}	1.956^{ab}	2.006^{a}	0.016	***<0.0001***	***<0.0001***	0.171
BWG, kg	1.298^{b}	1.359^{ab}	1.396^{a}	1.374^{a}	1.422^{a}	0.016	***<0.0001***	***<0.0001***	0.166
FI, kg	2.088	2.075	2.096	2.089	2.098	0.023	0.576	0.596	0.836
FCR, kg:kg	1.610^{a}	1.524^{b}	1.505^{bc}	1.522^{b}	1.476^{c}	0.009	***<0.0001***	***<0.0001***	***0.001***
14 to 35 d – Grower & Finisher Period									
35 d BW, kg	2.707^{b}	2.797^{ab}	2.839^{a}	2.852^{a}	2.857^{a}	0.027	***0.001***	***0.0001***	0.057
BWG, kg	2.126^{b}	2.216^{ab}	2.255^{a}	2.269^{a}	2.273^{a}	0.027	***0.001***	***0.0001***	0.058
FI, kg	3.513	3.465	3.490	3.489	3.487	0.039	0.942	0.811	0.651
FCR, kg:kg	1.666^{a}	1.583^{b}	1.550^{bc}	1.539^{c}	1.539^{bc}	0.011	***<0.0001***	***<0.0001***	***<0.0001***

[a-c]Means within a row that do not share a common superscript were deemed different ($P \leq 0.05$) by a Tukey's multiple comparison test.

[1]Values are least square means of 14 replicate pens per treatment.

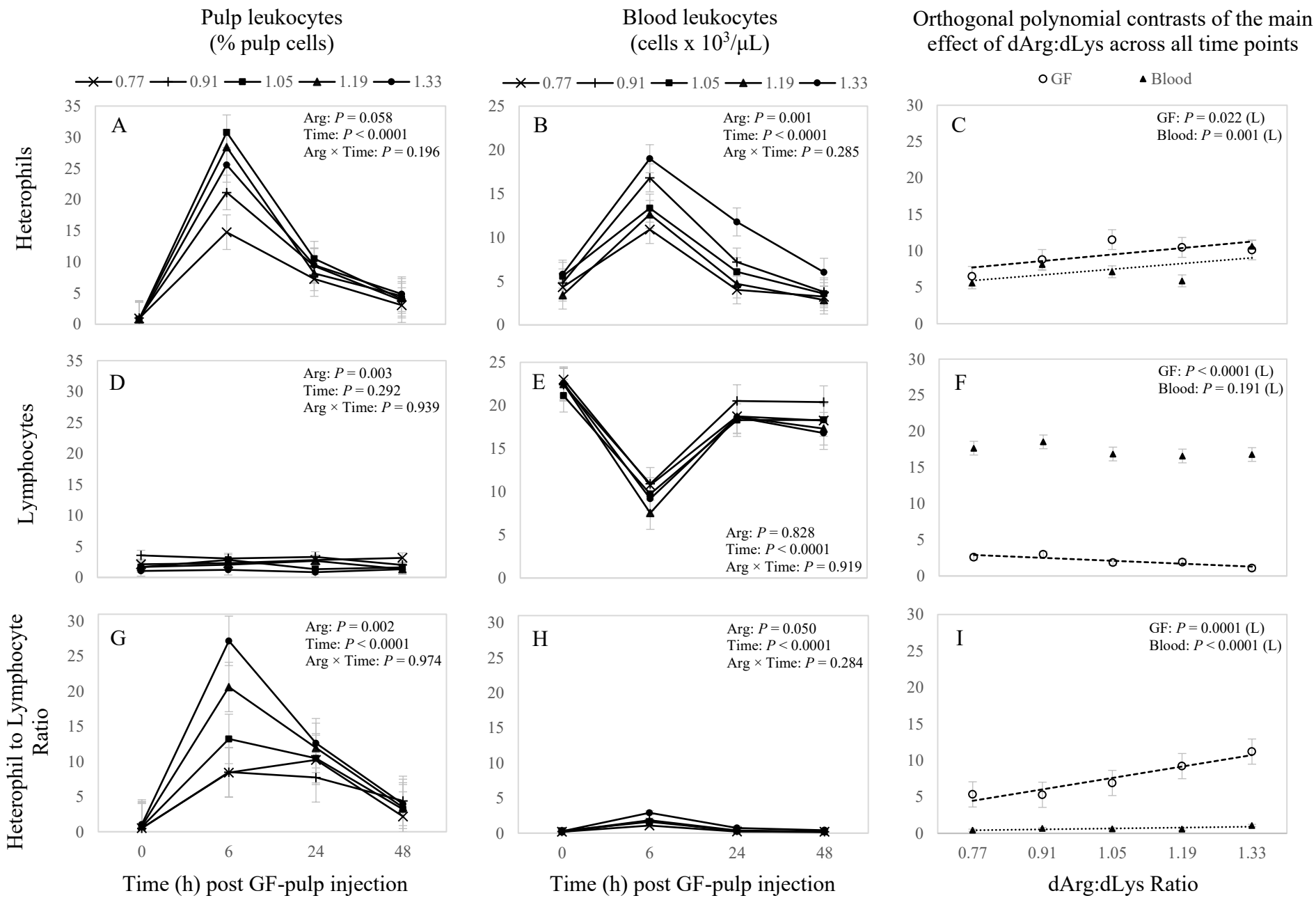

Pulp leukocytes
(% pulp cells)
Blood leukocytes
(cells x 10³/μL)
Orthogonal polynomial contrasts of the main effect of dArg:dLys across all time points
0.77 0.91 1.05 1.19 1.33
GF
Blood
Heterophils
Lymphocytes
Heterophil to Lymphocyte Ratio
A
Arg: P = 0.058
Time: P < 0.0001
Arg × Time: P = 0.196
B
Arg: P = 0.001
Time: P < 0.0001
Arg × Time: P = 0.285
C
GF: P = 0.022 (L)
Blood: P = 0.001 (L)
D
Arg: P = 0.003
Time: P = 0.292
Arg × Time: P = 0.939
E
Arg: P = 0.828
Time: P < 0.0001
Arg × Time: P = 0.919
F
GF: P < 0.0001 (L)
Blood: P = 0.191 (L)
G
Arg: P = 0.002
Time: P < 0.0001
Arg × Time: P = 0.974
H
Arg: P = 0.050
Time: P < 0.0001
Arg × Time: P = 0.284
I
GF: P = 0.0001 (L)
Blood: P < 0.0001 (L)
Time (h) post GF-pulp injection
Time (h) post GF-pulp injection
dArg:dLys Ratio

Figure 3.1. Heterophil and lymphocyte profiles of growing feather (**GF**) pulps and peripheral blood of Cobb 500 male broilers at 0, 6, 24, and 48 h after intradermal GF-pulp injection of lipopolysaccharide (**LPS**) at 40 d post-hatch (A-I). Broilers were fed increasing ratios of digestible arginine (**dArg**) to digestible lysine (**dLys**) from 14 to 42 d post-hatch, consisting of dArg:dLys 0.77, 0.91, 1.05, 1.19, and 1.33. Pulps of 12 GF from each of the 30 broilers (6 broilers per treatment) were intradermally injected with 10 µL of LPS (100 µg/mL; 1 µg/GF), and 2 GF per chicken were collected at 0 (before), 6, 24, and 48 h after GF-pulp injection and used to prepare pulp cell suspensions. Blood was collected with GF. GF-pulp and blood cell suspensions were subjected to direct, 2-3 color, immunofluorescent staining using fluorescently labeled mouse monoclonal antibodies specific to chicken leukocyte markers to identify lymphocytes (calculated by addition of T and B cells), and heterophil populations were identified within the $CD45^+$ pulp population and the $CD45^+CD41/61^-$ blood population based on size (FSC) and granularity (SSC) characteristics of leukocytes. Cell population analyses were conducted by flow cytometry. Data shown are mean leukocyte levels ± SEM for each dArg:dLys at each time point for both pulp (%) and blood (10^3 cells/µL) (A, B, D, E, G, H), while orthogonal polynomial contrasts (C, F, I) represent the main effect of dArg:dLys across all time points. Both linear (L) and quadratic (Q) contrasts were performed, but the most parsimonious model is shown.

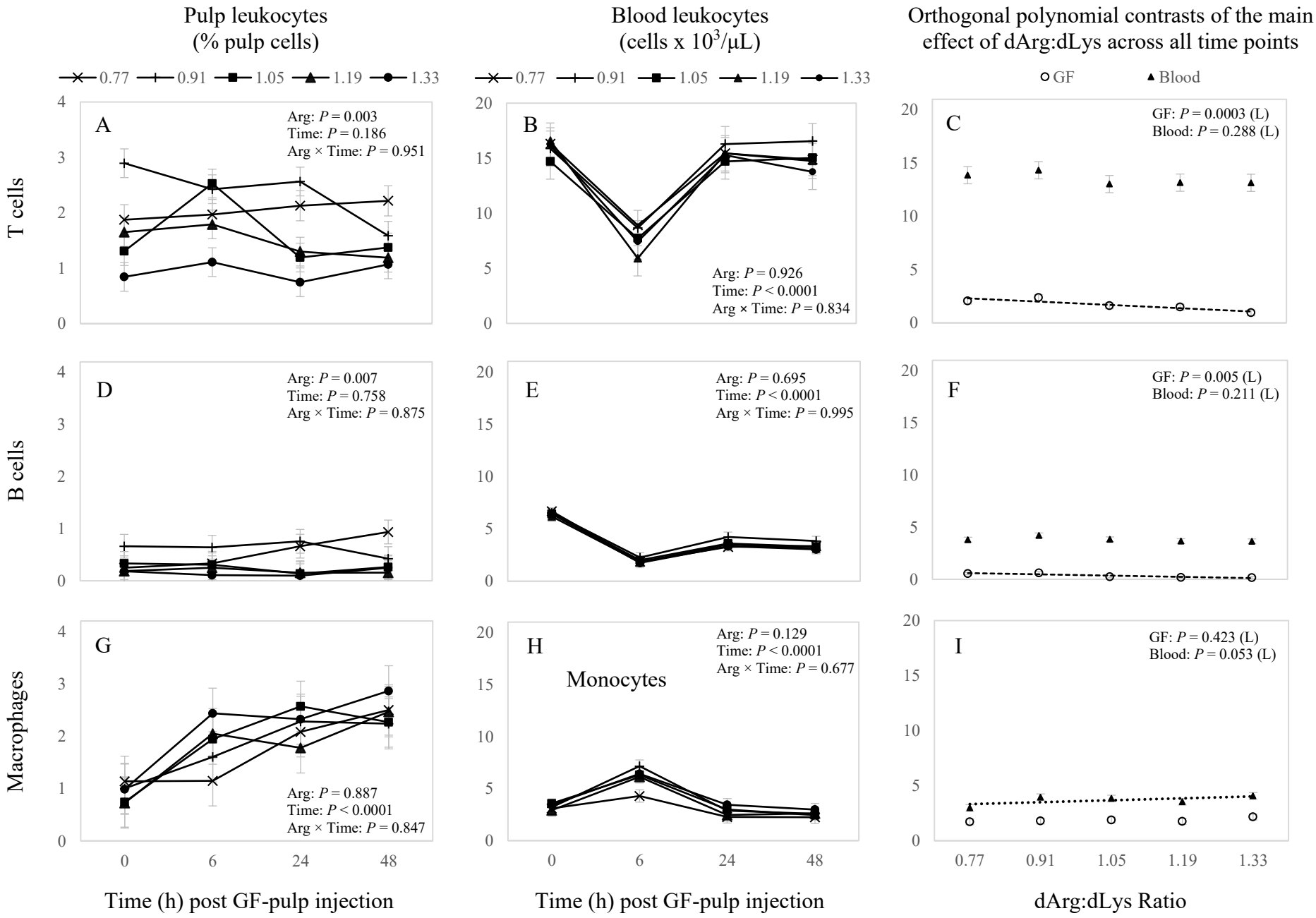

Pulp leukocytes
(% pulp cells)
Blood leukocytes
(cells x 10³/μL)
Orthogonal polynomial contrasts of the main effect of dArg:dLys across all time points
0.77
0.91
1.05
1.19
1.33
GF
Blood
T cells
B cells
Macrophages
A
Arg: P = 0.003
Time: P = 0.186
Arg × Time: P = 0.951
B
Arg: P = 0.926
Time: P < 0.0001
Arg × Time: P = 0.834
C
GF: P = 0.0003 (L)
Blood: P = 0.288 (L)
D
Arg: P = 0.007
Time: P = 0.758
Arg × Time: P = 0.875
E
Arg: P = 0.695
Time: P < 0.0001
Arg × Time: P = 0.995
F
GF: P = 0.005 (L)
Blood: P = 0.211 (L)
G
Arg: P = 0.887
Time: P < 0.0001
Arg × Time: P = 0.847
H
Monocytes
Arg: P = 0.129
Time: P < 0.0001
Arg × Time: P = 0.677
I
GF: P = 0.423 (L)
Blood: P = 0.053 (L)
Time (h) post GF-pulp injection
Time (h) post GF-pulp injection
dArg:dLys Ratio

Figure 3.2. T and B lymphocyte and macrophage/monocyte profiles of growing feather (**GF**) pulps and peripheral blood of Cobb 500 male broilers at 0, 6, 24, and 48 h after intradermal GF-pulp injection of lipopolysaccharide (**LPS**) at 40 d post-hatch (A-I). Broilers were fed increasing ratios of digestible arginine (**dArg**) to digestible lysine (**dLys**) from 14 to 42 d post-hatch, consisting of dArg:dLys 0.77, 0.91, 1.05, 1.19, and 1.33. Pulps of 12 GF from each of the 30 broilers (6 broilers per treatment) were intradermally injected with 10 µL of LPS (100 µg/mL; 1 µg/GF), and 2 GF per chicken were collected at 0 (before), 6, 24, and 48 h after GF-pulp injection and used to prepare pulp cell suspensions. Blood was collected with GF. GF-pulp and blood cell suspensions were subjected to direct, 2-3 color, immunofluorescent staining using fluorescently labeled mouse monoclonal antibodies specific to chicken leukocyte markers to identify specific cell populations, and analysis was conducted by flow cytometry. Data shown are mean leukocyte levels ± SEM for each dArg:dLys at each time point for both pulp (%) and blood (10^3 cells/µL) (A, B, D, E, G, H), while orthogonal polynomial contrasts (C, F, I) represent the main effect of dArg:dLys across all time points. Both linear (L) and quadratic (Q) contrasts were performed, but the most parsimonious model is shown.

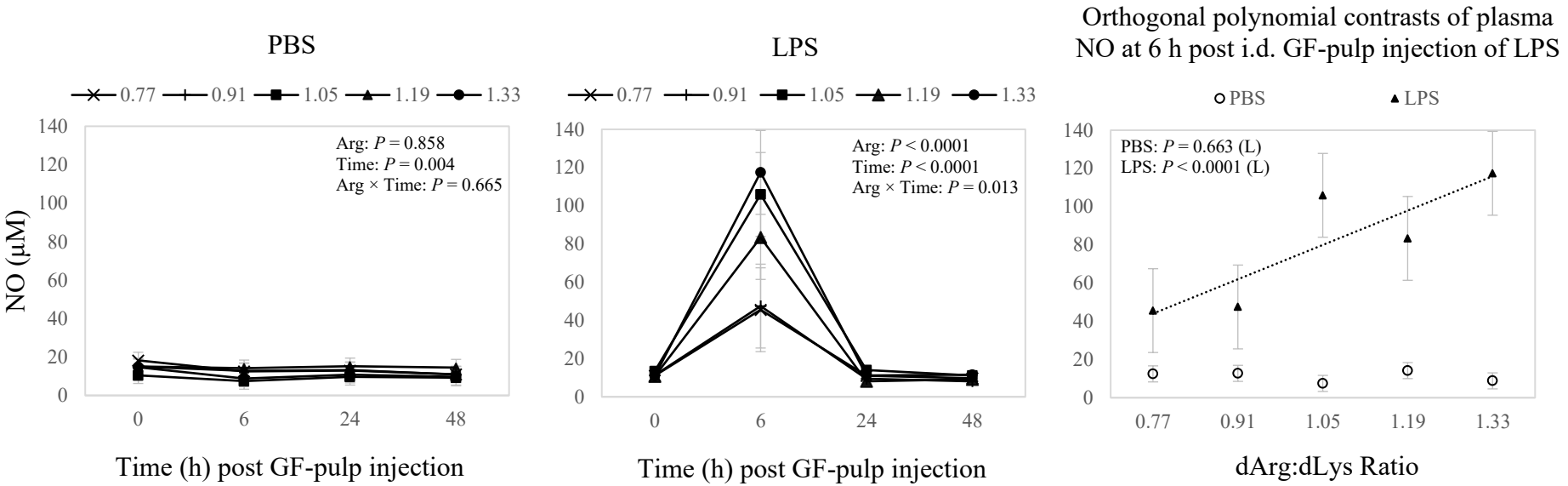


Figure 3.3. Nitric oxide (**NO**) concentration (μM) in the plasma of Cobb 500 male broilers at 0, 6, 24, and 48 h after intradermal growing feather (**GF**) pulp injection of either endotoxin-free phosphate-buffered saline (**PBS**) or lipopolysaccharide (**LPS**) at 40 d post-hatch. Broilers were fed increasing ratios of digestible arginine (**dArg**) to digestible lysine (**dLys**) from 14 to 42 d post-hatch, consisting of dArg:dLys 0.77, 0.91, 1.05, 1.19, and 1.33. Pulps of 12 GF from each of the 40 broilers (PBS, 2 broilers per treatment; LPS, 6 broilers per treatment) were intradermally injected with 10 μL of PBS or LPS (100 μg/mL; 1 μg/GF), respectively, and peripheral blood was collected at 0 (before), 6, 24, and 48 h after GF-pulp injection. Plasma was then isolated and used for determination of NO concentration by colorimetric (540 nm) microplate assay (Cayman Chemical). Data shown are mean NO concentrations ± SEM for each dArg:dLys at each time point for both PBS and LPS injections. Because of a significant interaction term in the two-way ANOVA for time and dArg:dLys, orthogonal polynomial contrasts were also performed for the simple effect of dArg:dLys at 6 h post-LPS injection. Both linear (L) and quadratic (Q) contrasts were performed, but the most parsimonious model is shown.

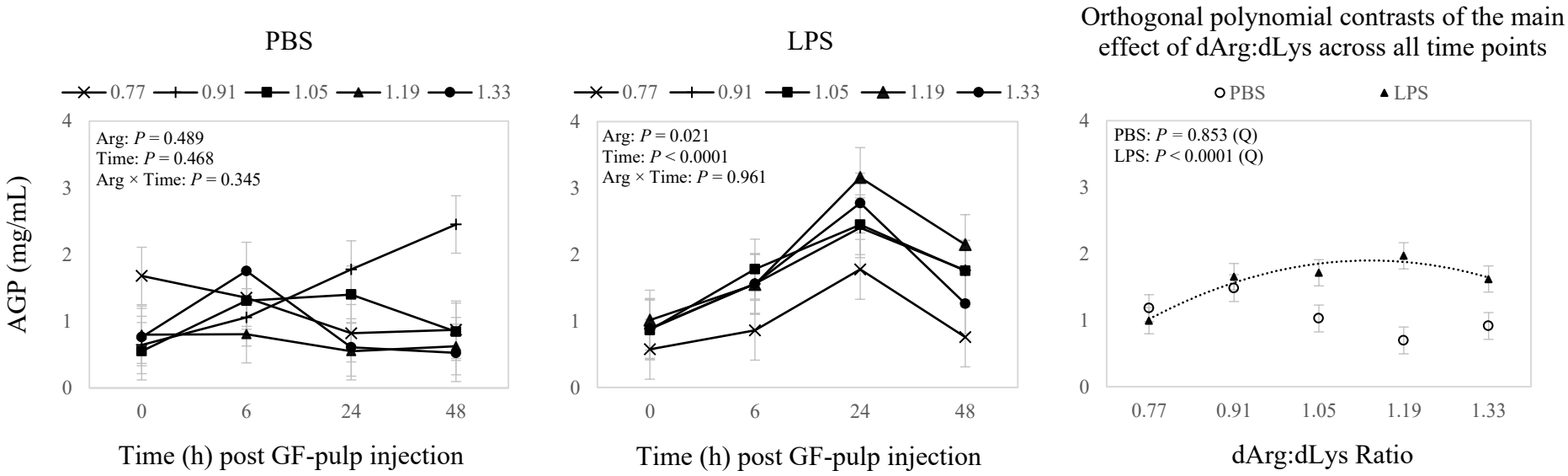


Figure 3.4. Alpha-1-acid glycoprotein (**AGP**) concentration (mg/mL) in the plasma of Cobb 500 male broilers at 0, 6, 24, and 48 h after intradermal growing feather (**GF**) pulp injection of endotoxin-free phosphate-buffered saline (**PBS**) or lipopolysaccharide (**LPS**) at 40 d post-hatch. Broilers were fed increasing ratios of digestible arginine (**dArg**) to digestible lysine (**dLys**) from 14 to 42 d post-hatch, consisting of dArg:dLys 0.77, 0.91, 1.05, 1.19, and 1.33. Pulps of 12 GF from each of the 40 broilers (PBS, 2 broilers per treatment; LPS, 6 broilers per treatment) were intradermally injected with 10 µL of PBS or LPS (100 µg/mL; 1 µg/GF), respectively, and blood was collected at 0 (before), 6, 24, and 48 h after GF-pulp injection. Plasma was then isolated and used for determination of AGP concentration by chicken AGP ELISA assay (Life Diagnostics). Data shown are mean AGP concentrations ± SEM for each dArg:dLys at each time point for both PBS and LPS injections. Orthogonal polynomial contrasts were also performed for the main effect of dArg:dLys across all time points post-LPS injection. Both linear (L) and quadratic (Q) contrasts were performed, but the most parsimonious model is shown.

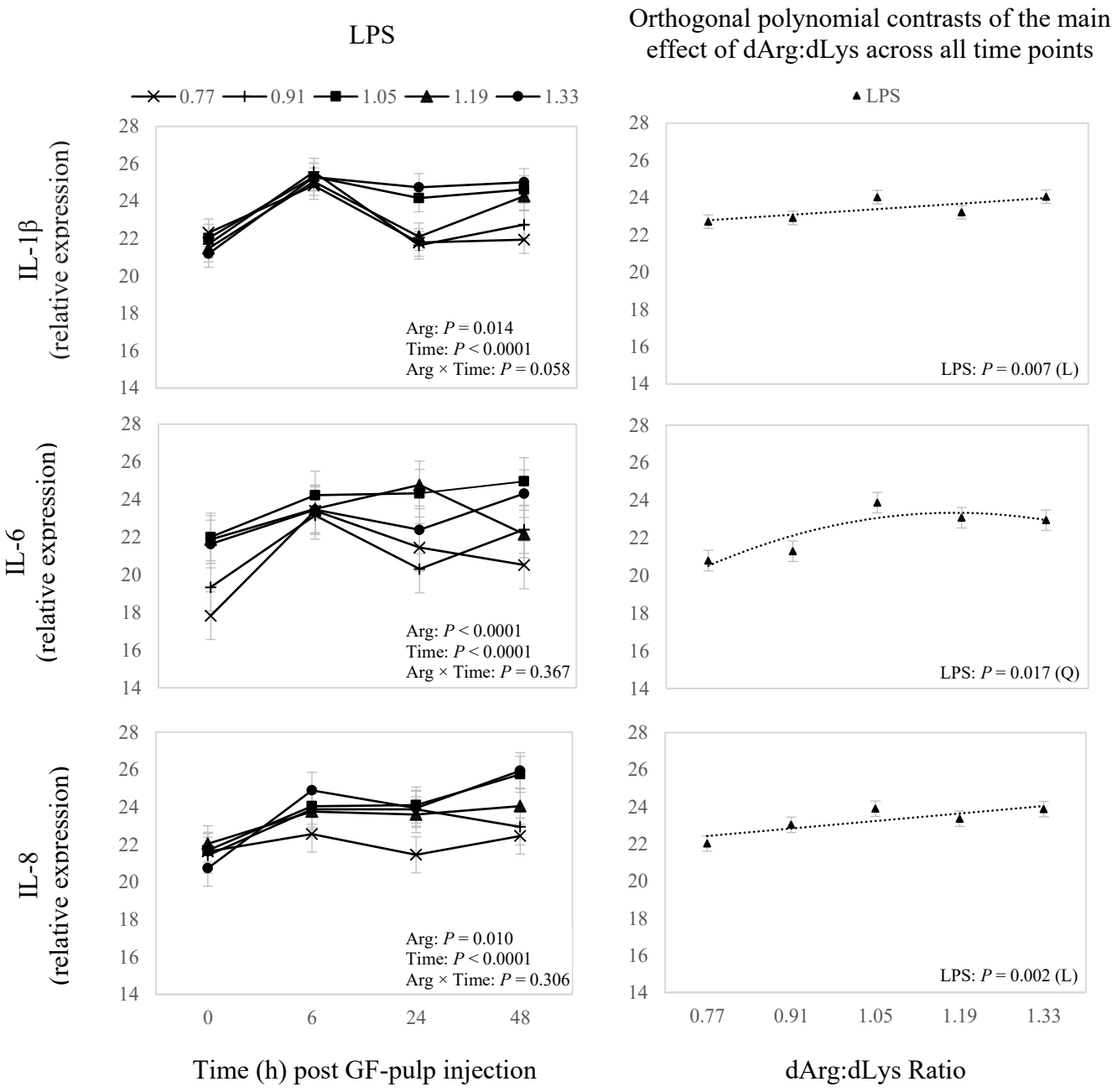


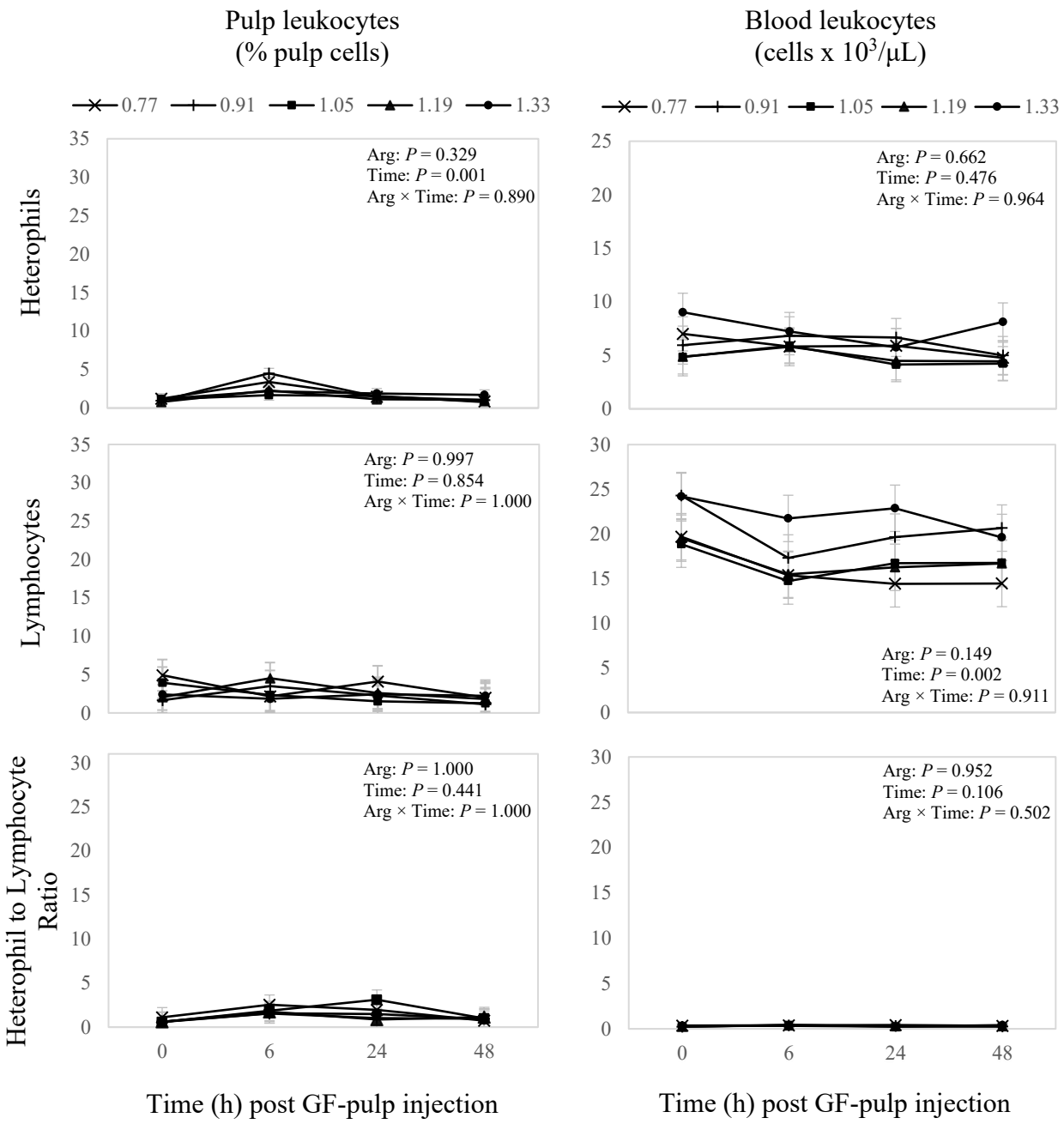
Figure 3.5. Relative expression of cytokine mRNA levels in growing feather (**GF**) pulps of Cobb 500 male broilers at 0, 6, 24, and 48 h after intradermal GF-pulp injection of lipopolysaccharide (**LPS**) at 40 d post-hatch. Broilers were fed increasing ratios of digestible arginine (**dArg**) to digestible lysine (**dLys**) from 14 to 42 d post-hatch, consisting of dArg:dLys 0.77, 0.91, 1.05, 1.19, and 1.33. Pulps of 12 GF from each of the 30 broilers (6 broilers per treatment) were intradermally injected with 10 µL of LPS (100 µg/mL; 1 µg/GF), and 2 GF per chicken were collected at 0 (before), 6, 24, and 48 h after GF-pulp injection and used for RNA isolation and relative gene-expression analysis by qRT-PCR. Analysis consisted of interleukin

1β (IL-1β), IL-6, and IL-8 (CXCL8). Data shown are mean relative mRNA expression (40-delta Ct) ± SEM for each dArg:dLys at each time point. Orthogonal polynomial contrasts were also performed for the main effect of dArg:dLys across all time points. Of the linear (L) and quadratic (Q) contrasts, the most parsimonious model is shown.

Supplemental Table 3.1. Primer and probe sequences for target genes, table adapted from French et al. (2020).

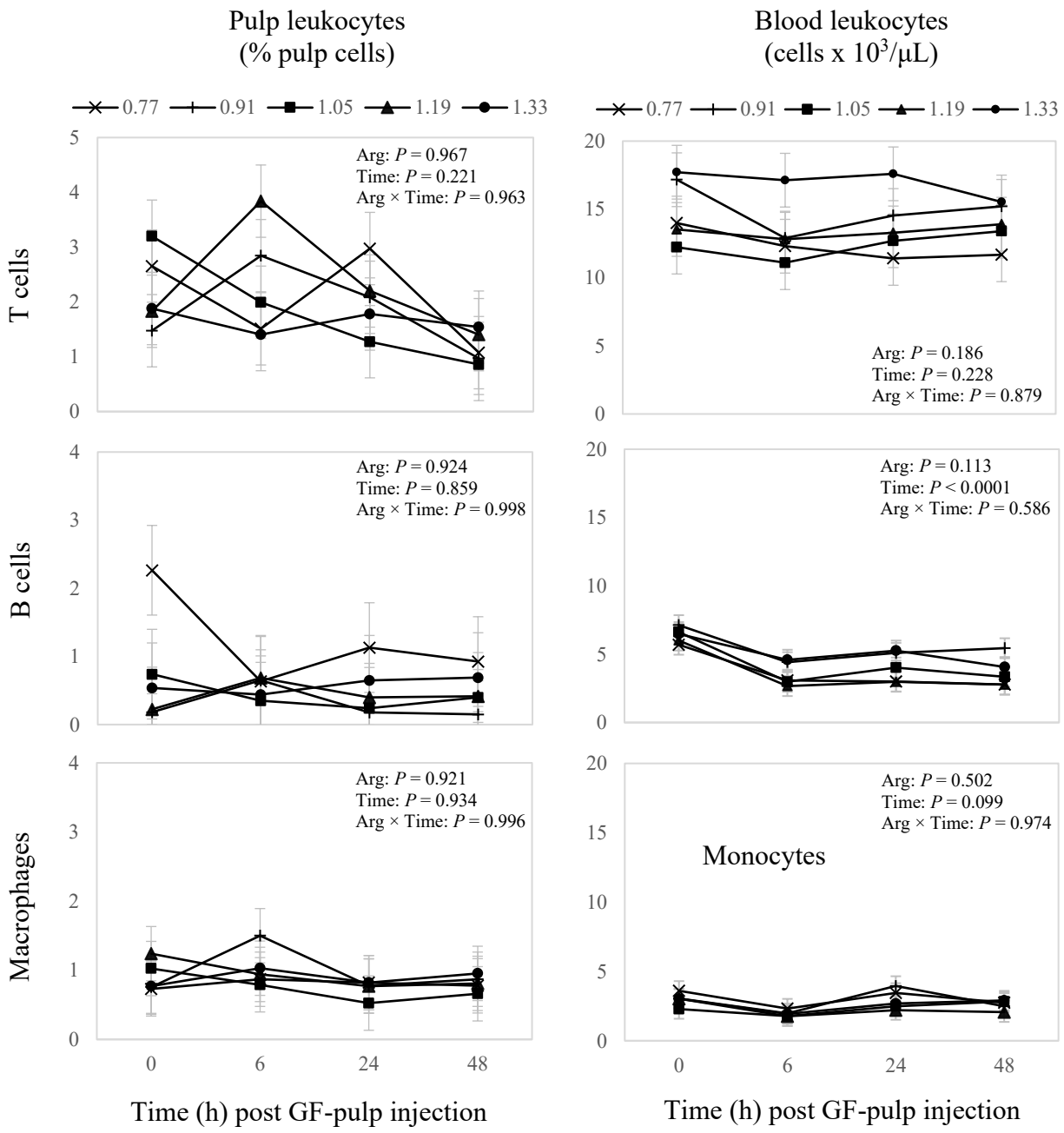
Target	Primer/probe	Sequences (5'–3')	Product size, bp	Accession No.
28s[1]	Forward	GGCGAAGCCAGAGGAAACT	62	FM165415
	Reverse	GACGACCGATTTGCACGTC		
	Probe	[FAM]TGGTGGAGGTCCGTAGCGGTCCT[TAM]		
IL-1β[1]	Forward	GCTCTACATGTCGTGTGTGATGAG	167	AJ245728
	Reverse	TGTCGATGTCCCGCATGA		
	Probe	[FAM]CCACACTGCAGCTGGAGGAAGCC[TAM]		
IL-6[2]	Forward	GCTCGCCGGCTTCGA	71	NM_204628.1
	Reverse	GGTAGGTCTGAAAGGCGAACAG		
	Probe	[FAM]AGGAGAAATGCCTGACGAAGCTCTCCA[TAM]		
IL-8/CXCL8[1]	Forward	GCCCTCCTCCTGGTTTCA	74	HM179639.1
	Reverse	TGGCACCGCAGCTCATT		
	Probe	[FAM]TGTCGCAAGGTAGGACGCTGGTAAAGA[TAM]		

[1]Sequences were from Smith et al. (2005).
[2]Sequences were from Kaiser et al. (2003).



Supplemental Figure 3.1. Heterophil and lymphocyte profiles of growing feather (**GF**) pulps and peripheral blood of Cobb 500 male broilers at 0, 6, 24, and 48 h after intradermal GF-pulp injection of sterile, endotoxin-free Dulbecco's phosphate buffered saline (**PBS**) at 40 d post-hatch. Broilers were fed increasing ratios of digestible Arg (**dArg**) to digestible lysine (**dLys**) from 14 to 42 d post-hatch, consisting of 0.77, 0.91, 1.05, 1.19, and 1.33 dArg:dLys. Pulps from 12 GFs from each other 10 broilers (2 broilers per treatment) were intradermally injected with 10 μL of PBS, and 2 GFs per chicken were collected at 0 (before), 6, 24, and 48 h after GF-pulp injection and used to prepare pulp cell suspensions. Blood was collected with GF. GF-pulp and

blood cell suspensions were subjected to direct, 2-3 color, immunofluorescent staining using fluorescently labeled mouse monoclonal antibodies specific to chicken leukocyte markers to identify lymphocytes (calculated by addition of T and B cells), and heterophil populations were identified within the $CD45^+$ pulp population and the $CD45^+CD41/61^-$ blood population based on size (FSC) and granularity (SSC) characteristics of leukocytes. Cell population analyses were conducted by flow cytometry. Data shown are mean leukocyte levels ± SEM for each dArg:dLys at each time point for both pulp (%) and blood (10^3 cells/μL).



Supplemental Figure 3.2. T and B lymphocyte and macrophage/monocyte profiles of growing feather (**GF**) pulps and peripheral blood of Cobb 500 male broilers at 0, 6, 24, and 48 h after intradermal GF-pulp injection of sterile, endotoxin-free Dulbecco's phosphate buffered saline (**PBS**) at 40 d post-hatch. Broilers were fed increasing ratios of digestible Arg (**dArg**) to digestible lysine (**dLys**) from 14 to 42 d post-hatch, consisting of 0.77, 0.91, 1.05, 1.19, and 1.33 dArg:dLys. Pulps from 12 GFs from each other 10 broilers (2 broilers per treatment) were intradermally injected with 10 μL of PBS, and 2 GFs per chicken were collected at 0 (before), 6, 24, and 48 h after GF-pulp injection and used to prepare pulp cell suspensions. Blood was

collected with GF. GF-pulp and blood cell suspensions were subjected to direct, 2-3 color, immunofluorescent staining using fluorescently labeled mouse monoclonal antibodies specific to chicken leukocyte markers to identify specific cell populations, and analysis was conducted by flow cytometry. Data shown are mean leukocyte levels ± SEM for each dArg:dLys at each time point for both pulp (%) and blood (10^3 cells/μL).

IV. DIETARY ARGININE RESPONSES OF ROSS 708 AND COBB 500 BROILERS REARED UNDER CYCLIC ELEVATED TEMPERATURES

ABSTRACT

The objective of this study was to evaluate the effects of dietary Arg ratio on the performance and processing characteristics of broilers subjected to a cyclic heat stress (**HS**) challenge. A total of 1,200 male Ross 708 (Experiment 1) and 1,104 Cobb 500 (Experiment 2) broiler chicks were fed a common starter and grower diet to 27 d before being fed 1 of 6 target dietary digestible Arg:Lys ratios (0.80, 0.92, 1.04, 1.16, 1.28, and 1.40) across both finisher 1 (27 to 38 d) and finisher 2 (38 to 46 d) phases. At placement, barn temperature was set to 32°C and decreased to maintain bird comfort until initiation of the HS challenge at 28 d. From 28 to 47 d, barn temperature was maintained at 32°C for 12 h daily (07:30 to 19:30 h) and reduced to 24°C each night. At 32, 39, and 46 d, cloacal temperatures were measured in 2 birds per pen at approximately 06:30 h (thermoneutral; **TN**) and 14:30 h (HS). At 48 d, 8 birds per pen were processed and deboned to determine carcass as well as parts weights and yields. Data were analyzed by one-way ANOVA with pen location as a random blocking variable. Linear and quadratic contrasts of dArg:dLys ratio were also performed. Statistical significance was considered at $P \leq 0.05$. Feed conversion ratio improved linearly with increasing dArg:dLys during the finisher 1 phase in both experiments and in the cumulative finisher phase in experiment 1 ($P < 0.05$). Cloacal temperatures were approximately 1.6°C higher during HS than during the TN period at each time point and were influenced by dietary Arg in experiment 1 only ($P < 0.05$), quadratically at 32 and 39 d but linearly at 46 d. For processing characteristics, there was a linear increase ($P < 0.05$) in chilled carcass (Exp. 1), tender (Exp. 1), thigh (Exp. 2), and

drum (Exp. 2) weights with increasing Arg, with quadratic responses ($P < 0.05$) in breast fillet (Exp. 1), total breast (Exp. 1), and fat pad (Exp. 2) weights and yields. Overall, the results of this study indicate that in addition to improving feed efficiency and 48 d processing yields, dietary Arg may also influence core body temperature in a dose-dependent manner in certain broiler genotypes that are subjected to a cyclic HS challenge.

INTRODUCTION

Due to its deleterious effects on bird behavior, physiology, welfare, gut health, and growth performance, heat stress (**HS**) is one of the main environmental stressors challenging poultry production worldwide (Lara and Rostagno, 2013; Brugaletta et al., 2022). Heat stress occurs when the amount of heat energy produced by the animal exceeds the amount of energy dissipated from its body into the surrounding environment, and due to the lack of sweat glands and a limited unfeathered body surface area to facilitate heat loss, poultry can be particularly sensitive to this condition (Lara and Rostagno, 2013; Zhang et al., 2017). Modern broilers, which have been genetically selected to be fast growing and highly efficient, are even more susceptible to HS due to their extremely high metabolic rates (Settar et al., 1999; Syafwan et al., 2011).

In hot temperatures, birds alter their behavior, metabolism, and physiological homeostasis to improve thermoregulation (Lara and Rostagno, 2013). As such, common consequences of HS include decreased feed intake (**FI**), panting, systemic inflammation, reduced skeletal muscle accretion, fat accumulation, respiratory alkalosis, and oxidative stress (Brugaletta et al., 2022). In addition, HS causes gastrointestinal tract dysfunction, including paracellular barrier failure, dysbiosis, enteritis, and digestive and absorptive disorders (Quinteiro-Filho et al., 2012; Brugaletta et al., 2022; Teyssier et al., 2023). Decreased FI to limit metabolic heat

production is the main factor impacting broiler performance (accounting for ~80% of the body weight gain (**BWG**) reduction during constant HS); however, the remainder of the detriment can be attributed to direct effects of HS per se (Syafwan et al., 2011; De Souza et al., 2016; Teyssier et al., 2022a,b). Research has shown that adjustment of feeding practices and nutritional programs may help mitigate these negative effects, and thus, have a critical role to play in broiler husbandry (Syafwan et al., 2011; Wasti et al., 2020; Teyssier et al., 2022b).

One such nutritional alteration is to modify the dietary inclusion of functional amino acids (**AA**) such as arginine (**Arg**). Arginine is an essential AA for poultry and is a precursor for many important molecules including nitric oxide (**NO**), polyamines, creatine, glutamate, and proline (Wu et al., 2009; Castro and Kim, 2020). Thus, Arg has several secondary functions in health and growth (Castro and Kim, 2020) that could be particularly beneficial for broilers experiencing HS. For example, NO is a potent effector molecule shown to influence thermoregulatory vasodilation (Taylor and Bishop, 1993; Canini et al., 2001; Uyanga et al., 2021). It is also directly and indirectly involved in antioxidant function, which could attenuate the overproduction of reactive oxygen species and oxidative injury caused by high ambient temperatures (Rubbo et al., 1996; Atakisi et al., 2009; Tan et al., 2010; Shan et al., 2013). Furthermore, polyamines can help to maintain gastrointestinal barrier function and benefit mucosal integrity and repair after damage (Tan et al., 2024). It has been hypothesized the Arg requirement of broilers under HS may increase to support these functions. Therefore, the objective of this study was to evaluate the response of increasing digestible Arg:Lys ratios for broilers subjected to cyclic HS to evaluate the resulting effects on growth performance, meat yield, core body temperature, and indicators of Arg metabolism.

MATERIALS AND METHODS

The protocol for this study was reviewed and approved by the Auburn University Institutional Animal Care and Use Committee (IACUC; animal use protocol #2023-5181). Experiment 1 and 2 were executed concurrently and only differ in procedure where indicated.

Bird Husbandry

A total of 1,400 male YP × Ross 708 broiler chicks (Experiment 1) and 1,400 male MV × Cobb 500 broiler chicks (Experiment 2) were transported from commercial hatcheries to the Auburn University Poultry Research Farm in Auburn, AL. Chicks were weighed by group to determine the average overall BW of each genotype, and 1,296 of each genotype (2,592 total) were allotted to 48 floor pens (27 chicks per pen, 96 total pens) such that all group weights were within 2% of each overall expected group weight. At 27 d, bird number was reduced to 25 and 23 birds per pen for the Ross and Cobb genotype, respectively, and birds were reallotted to pens within genotype based on BW to reduce variability. Bird number per pen differed due to the Cobb genotype experiencing higher than expected mortality throughout the entirety of the trial (cumulative mortality of 12.3% in the pre-experimental phase and 13.5% in the experimental phase, not influenced by treatment ($P = 0.210$; data not shown)).

Pens measured 2.79 m^2, which provided the minimum floor space required (0.11 m^2) for broilers up to 8 wk of age as described by The Ag Guide (Scanes et al., 2020). The stocking density at the end of the trial based on average 46 d BW was therefore approximately 26.5 kg/m^2 for the Ross genotype and 25.9 kg/m^2 for the Cobb genotype. All pens contained used pine litter top-dressed with fresh shavings and were equipped with a single commercial-type pan feeder and nipple waterers to provide free access to feed and clean water throughout the trial. Supplemental waterers and TurboGrow® (AMT, Inc., Laurens, SC, USA) chick pan feeders were placed in

each pen from 0 to 7 d post-hatch to facilitate access to feed and water for young chicks. Birds were monitored multiple times daily for any signs of distress (lethargy, impaired appetite, dehydration, etc.) and any birds that overtly exhibited these signs were euthanized using CO_2 inhalation or cervical dislocation. Light was provided for 23L:1D at 100% intensity during 0 to 7 d and for 18L:6D at 5-10 lux from 8 d until the end of the trial.

Experimental Diets

Broilers were fed diets in four phases that included a starter phase (0 to 17 or 0 to 14 d for Experiment 1 (Ross) and Experiment 2 (Cobb), respectively), a grower phase (17 to 27 or 14 to 27 d for Experiment 1 and Experiment 2, respectively), a finisher 1 phase (27 to 38 d), and a finisher 2 phase (38 to 46 d). The starter diet was pelleted and crumbled, while the grower and finisher feeds were fed as pellets. Prior to formulation, main ingredients were analyzed for total AA profile and CP content by wet chemistry analysis to improve accuracy of feed formulation. The common starter and common grower diets were corn and soybean meal based and formulated differently for each genotype to meet or exceed their respective primary breeder nutrient recommendations (Table 4.1; Aviagen, 2022; Cobb-Vantress, 2022). The finisher 1 and finisher 2 formulations were the same for both genotypes and were designed to be nutritionally complete and meet industry specifications except for Arg concentration (Table 4.2). The basal finisher diets included distiller's dried grains with solubles and corn gluten meal in order to achieve the lowest target Arg ratio, and crystalline L-Arg (CJ Bio, Seoul, Korea) was then added to the basal diet at the expense of an inert filler to achieve six dietary digestible Arg:Lys ratios of 0.80, 0.92, 1.04, 1.16, 1.28, 1.40. These ratios were selected to range from below to well above the primary breeder recommendation for Arg during the experimental ages. Complete feeds from each growth phase were sampled and analyzed for nutrient composition, and all feeds were

manufactured at the Auburn University feed mill. Diet samples were analyzed to determine nitrogen (**N**) for CP via combustion (Method 990.03; AOAC International, 2007), total AA concentrations (Method 982.30 E [a, b, and c]; AOAC International, 2007), as well as free Arg concentration (Method 999.13; AOAC International, 2007) at the Agricultural Experiment Station Chemical Laboratories at the University of Missouri-Columbia.

Rearing Temperatures and Cyclic Heat Stress Model

Barn temperature was set to 32°C and decreased gradually throughout the trial to maintain bird comfort. At 28 d, a cyclic HS program was employed where barn temperature was maintained at 32°C for 12 h daily (07:30 to 19:30 h) and reduced to 24°C each night. This model was intended to mimic the cyclic, diurnal pattern of heat stress experienced by broilers reared in temperate regions during summer and in tropical climates year-round. Moderate HS during the hot period was confirmed by increased panting, modified bird behavior (leg extension), and an increase in average core body temperature during times when heat was applied, without an increase in mortality. Throughout the trial, maximum and minimum barn temperature and humidity were recorded every 10 min by HOBO® temperature loggers, which were evenly distributed throughout the house and placed at bird level. Recorded average relative humidity during the trial was 52%.

Growth Performance

Feeder and bird pen weights were recorded at 0, 14 (Experiment 2 only), 17 (Experiment 1 only), 27, 38, and 46 d post-hatch to determine BW, BWG, FI, and mortality-corrected feed conversion ratio (**FCR**) for each feeding phase and cumulatively. Feed intake was calculated based on number of bird days to account for mortality.

Core Body Temperature Measurement

Core body temperature of the same two marked birds per pen were measured at 32, 39, and 46 d post-hatch at both 06:00 h (before daily heat was applied) and 14:00 h (during peak daily heat application) using a DeltaTrak (Pleasanton, CA, USA) model 11064 blunt probe digital thermometer with an accuracy of ± 0.5°C. At each measurement, birds were gently restrained by both feet with their breast supported and held at an approximately 45 degree angle, while trained personnel gently inserted the thermometer into the cloaca of the chicken for about 10 s at a depth of 25 mm.

Blood and Tissue Sample Collection

Experiment 1 (Ross 708). At the end of the finisher 1 phase (38 d), approximately 2-3 mL of blood was collected from the brachial wing vein of one marked bird per pen to determine plasma nitric oxide (**NO**), alpha-1-acid glycoprotein (**AGP**), and free AA concentrations. Blood was collected into BD Vacutainer™ plastic tubes spray-coated with K_2EDTA as an anticoagulant, and centrifuged at 400 × *g* at 4°C for 10 min to isolate plasma. Plasma samples were stored at -80°C for later analysis. At the end of the finisher 2 phase (46 d), approximately 5 mL of blood was collected postmortem from the femoral vessels of the same marked bird using identical processing methods to 38 d. Blood was used for plasma NO, AGP, and free AA concentrations, as well as determination of the concentrations and proportions of heterophils and lymphocytes in the whole blood.

Experiment 2 (Cobb 500). Approximately 5 mL of blood was collected at the end of the finisher 2 phase (46 d) using the same methods as described for Experiment 1. Blood samples were used to determine plasma concentrations of NO and AGP.

Processing

Eight birds per pen were randomly selected, wing-banded, and marked for processing which occurred at 48 d. Each marked bird was individually weighed the afternoon before the day of processing and fasted for approximately 11 h overnight before being transported to the Fortenberry Processing Plant located on site. At the plant, birds were humanely euthanized, processed, and mechanically eviscerated before being placed in ice water for about 3 h prior to recording cold carcass and abdominal fat pad weights. Carcasses were again placed in ice water overnight. At 49 d, carcasses were hand-deboned by professional deboners, and the weights of the pectoralis major (P. major) and minor (P. minor) muscles, wings, thighs, and drums were recorded. Total breast meat (**TBM**) was calculated as the sum of the P. major and P. minor weights, and the yield of each part was determined by division of the part weight by the individual live weight taken the afternoon before the day of processing.

Plasma Nitric Oxide and Alpha-1-Acid Glycoprotein Analyses

Concentration of NO (μM) in plasma samples was determined using a commercial colorimetric assay kit following the manufacturer's protocol (Cayman Chemical Company, Ann Arbor, MI). As the final in vivo oxidation products of NO, the sum of both nitrate (NO_3^-) and nitrite (NO_2^-) were used to represent total NO. Prior to analysis, plasma samples were filtered through pre-rinsed Amicon® Ultra 30,000 MWCO filters (MilliporeSigma, Burlington, MA) to remove interfering proteins with a molecular weight greater than 30 kilodaltons, and were then diluted 1:2 with assay buffer. Concentration of AGP (mg/mL) was determined using a chicken AGP sandwich ELISA according to the manufacturer's protocol (Life Diagnostics, West Chester, PA), and samples were diluted 1:10,000 with assay diluent prior to conducting the assay.

Plasma Amino Acid Analyses

Analysis of plasma AA was completed using ultra high-performance liquid chromatography. Prior to analysis, plasma samples were thawed and 100 µL of each sample was deproteinated with an equal volume of 10% w/v trichloroacetic acid, incubated for 10 min on ice, and centrifuged at 12,000 × *g* for 20 min at 4°C. Then, 10 µL of the supernatant was derivatized using 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate reagent following the methods of Outlaw et al. (2023). The AA in the supernatant were separated using an Acquity UPLC H-Class Plus chromatography instrument (Waters Corporation, Milford, MA) equipped with a Waters Acquity UPLC BEH C18 column (1.7 µm, 2.1 mm × 150 mm; Waters Corporation, Milford, MA) and detected by fluorescence to determine the concentrations of each individual AA. Waters® Empower™ 3 chromatography software was used to collect and process data.

Leukocyte Profiles

Whole blood samples were measured using the Cell-Dyn 3500 blood analysis system (Abbott Diagnostics, Abbott Park, IL), which was standardized for chicken blood. The analysis uses electronic impedance and laser-light scattering to estimate the concentration and proportion of heterophils and lymphocytes in the sample. The heterophil to lymphocyte (**H:L**) ratios were calculated by dividing the estimates of heterophils by that of total lymphocytes.

Statistical Analyses

For each experiment, data were analyzed by one-way ANOVA for a randomized complete block design with pen as the experimental unit and pen location as a random blocking factor, with 8 replicate pens per treatment. A Tukey's HSD test was used to separate means, while polynomial contrasts were used to determine any linear or quadratic trends in increasing dArg:dLys concentration (0.80 to 1.40 dArg:dLys) for each dependent variable. Variables with

significant polynomial responses were subsequently subjected to regression analyses to properly evaluate dose responses to dietary Arg. In all cases, statistical significance was determined at $P \leq 0.05$, and data were analyzed using JMP Pro 17.2 software (SAS Institute, Cary, NC).

RESULTS

Diet Analyses

Calculated values for the common starter and grower diets are shown in Table 4.1, and calculated and analyzed values for the experimental finisher 1 and finisher 2 diets are shown in Table 4.2. As anticipated, analyzed total and free Arg concentration increased proportionally as calculated digestible Arg values increased. Free Arg concentration in the finisher 1 0.80 dArg:dLys diet was 0.03%, and concentration increased by 0.10% on average in each subsequent diet up to 0.55% for the 1.40 dArg:dLys diet. Similarly, the finisher 2 0.80 dArg:dLys diet started at 0.04%, increasing by 0.09% on average in each subsequent diet up to 0.50% for 1.40 dArg:dLys.

Growth Performance

Experiment 1 (Ross 708). Average chick weight at placement for the Ross 708 genotype was 0.037 kg with a 0 to 27 d BWG of 1.447 kg before the start of the experimental phase. Increasing the dietary dArg:dLys ratio from 0.80 to 1.40 linearly improved FCR during the finisher 1 ($P = 0.001$) and cumulative finisher ($P = 0.012$) phases (Table 4.3). The lowest numerical values for FCR during the finisher 1 phase was observed at a dArg:dLys ratio of 1.28, which was 11 points lower than the FCR of birds fed a dArg:dLys ratio of 0.80. No other effects of Arg on growth performance were observed.

Experiment 2 (Cobb 500). Average chick weight at placement for the Cobb 500 genotype was 0.039 kg with a 0 to 27 d BWG of 1.676 kg before the start of the experimental phase. Increasing the dArg:dLys ratio from 0.80 to 1.40 linearly improved FCR during the finisher 1 phase ($P = 0.018$) and tended to linearly improve the cumulative FCR ($P = 0.056$; Table 4.3). Despite being only a numeric difference, the largest effect size of improvement was 20 points from the highest to lowest FCR value in the cumulative phase (0.80 versus 1.28 dArg:dLys, respectively). No other significant effects of Arg on growth performance during this period were observed.

Cloacal Temperatures

Experiment 1 (Ross 708). During the hot period (14:00 h), dietary dArg:dLys influenced cloacal body temperature in a dose-dependent manner at all measured time points (Table 4.4). At both 32 and 39 d, increasing dietary Arg quadratically decreased ($P = 0.022$ and 0.021, respectively) cloacal temperature, with the numerically lowest body temperature measured in birds fed a dArg:dLys ratio of 1.16. However, at 46 d the trend was linear ($P = 0.011$) with a tendency for a quadratic effect ($P = 0.087$). The lowest body temperature at 46 d was measured in birds fed 1.28 dArg:dLys. Despite effects of Arg on cloacal body temperature during the hot period, there was no significant effect of dArg:dLys ratio during either the thermoneutral period (06:00 h) or on the difference in body temperature between the hot and thermoneutral periods.

Experiment 2 (Cobb 500). At 32, 39, and 46 d, there were no significant effects of dArg:dLys ratio on the cloacal core body temperatures measured during either the thermoneutral (06:00 h) or hot (14:00 h) period, or on the difference in core body temperatures measured between the two periods (Table 4.4).

Plasma Concentrations of Metabolites

Experiment 1 (Ross 708). There was no effect ($P > 0.05$) of dArg:dLys ratio on the proportion or concentration of heterophils, lymphocytes, or the heterophil to lymphocyte ratio observed at 46 d (Table 4.5). Similarly, there was no effect ($P > 0.05$) of Arg on the plasma concentrations of NO or AGP measured at either 38 or 46 d post-hatch (Tables 4.6 and 4.7). However, dArg:dLys ratio did have a significant effect on the concentrations of several AA measured at both 38 and 46 d (Tables 4.6 and 4.7). Most notably, plasma concentrations of Arg, ornithine (**Orn**), and citrulline (**Cit**) were consistently increased as the ratio of dArg:dLys in the diet increased ($P < 0.05$). Additionally, concentrations of serine (**Ser**), glutamine (**Gln**), glycine (**Gly**), and threonine (**Thr**) were consistently affected by Arg ratio at both time points in a dose-dependent manner ($P < 0.05$).

Experiment 2 (Cobb 500). At 46 d post-hatch, dArg:dLys ratio had no effect ($P > 0.05$) on either the proportions or concentrations of heterophils or lymphocytes in the blood, or the heterophil to lymphocyte ratio (Table 4.5). There was also no effect of Arg on plasma NO concentration ($P = 0.844$) or AGP concentration ($P = 0.106$) (data not shown). The average NO concentration in this experiment was 24.350 ± 10.215 μM and the average AGP concentration was 0.355 ± 0.068 mg/mL.

Processing

Experiment 1 (Ross 708). Increasing dArg:dLys significantly influenced several 48 d processing characteristics in a dose-dependent manner (Table 4.8). Chilled carcass yield increased linearly ($P = 0.006$) by 0.95% from the lowest to highest Arg concentration, and there was also a linear improvement in tender yield ($P = 0.038$). There was a quadratic response in chilled carcass weight ($P = 0.034$), breast fillet weight ($P = 0.007$) and yield ($P = 0.018$), tender

weight (P = 0.040), total breast weight (P = 0.009) and yield (P = 0.023), and drum yield (P = 0.031) as dietary Arg increased. For all parts except the drum, the increase in dArg:dLys ratio had a positive quadratic effect on the individual parts weights or yields, reaching maximum numeric values at the dArg:dLys ratio of either 1.16 or 1.28.

Experiment 2 (Cobb 500). There was a linear increase in the absolute weight of thighs (P = 0.009) and drums (P = 0.035) with increased dArg:dLys ratio, as well as a quadratic response in fat pad weight (P = 0.005) and yield (P = 0.019) at 48 d (Table 4.9).

DISCUSSION

The current study was designed to evaluate the performance and processing characteristics of two commercial broiler genotypes fed increasing concentrations of Arg during a cyclic HS challenge. Increasing the dArg:dLys ratio from 0.80 to 1.40 during the finisher period did not result in any significant differences in BWG or FI for heat-stressed broilers of either genotype; however, there was a consistent linear improvement in FCR during the finisher 1 phase in both experiments and in the cumulative finisher phase in experiment 1. These linear improvements indicate that the Arg ratios in the titration were not high enough to achieve a breakpoint for the estimate of a requirement. However, these results do confirm that FCR is responsive to dArg:dLys ratios in heat-stressed broilers in a dose-dependent manner. In our experiments, improvements in FCR from the highest to lowest numeric value for each phase ranged from 9 to 18 points.

The results of other studies that have evaluated broiler performance responses to Arg under HS vary. Brugaletta et al. (2023a) reported no performance benefits of 1.20% total Arg:Lys compared to the recommendation (1.09% Arg:Lys) during the finisher phase, while

Nassar et al. (2022) and Abd El-Maged and Desoky (2023) report an improvement in BWG and FCR in various growth periods using 0.5-2.0 g/kg Arg supplementation to an unspecified basal diet Arg concentration and a 1.17% total Arg:Lys basal diet, respectively. However, several published works show that feeding broilers higher dietary Arg concentrations during periods of either cyclic or constant HS may result in FCR improvements only (Mendes et al., 1997; Brake et al., 1998; Lee et al., 2024; Oliveira et al., 2024), which is in agreement with the results of our current study. Interestingly, Brake et al. (1998) reared broilers from 20 to 41 d in either 20°C or 31°C with a total Arg:Lys ratio of 1.09% or 1.36%, and found that BWG was increased in broilers fed 1.36% Arg in the 20°C environment but that the trend was only numeric for the broilers reared in constant HS (31°C). For FCR, broilers in constant HS had improved FCR with 1.36% Arg but those reared at 20°C were not different. These data suggest that the performance benefits of Arg may be sensitive to rearing temperature. As many studies have shown that dietary Arg supplementation can improve BWG and FCR under thermoneutral conditions (Xu et al., 2018a; Zampiga et al., 2018; Castro et al., 2019; Liu et al., 2019; Sirathonpong et al., 2019; Brugaletta et al., 2023a,b), perhaps the mechanisms by which Arg improves BWG are physiologically affected by HS, whereas those that improve FCR are not. Most likely, however, protein accretion to support BWG is impacted by HS per se and thus can be independent of diet (Teyssier et al., 2022a); therefore, more dietary Arg would not benefit BWG in this case.

It is also possible that suboptimal conditions such as HS result in a metabolic prioritization of Arg for other body functions like NO and antioxidant production, which could enhance feed efficiency despite no increases in muscle protein accretion and growth per se. Indeed, NO has been demonstrated to be a potent regulator of both the central and peripheral control of the physiological temperature response (Gerstberger, 1999; Branco et al., 2014;

Uyanga et al., 2022). Centrally, it has been suggested that NO can influence sympathetic tone by acting on various brain sites, and peripherally, NO is implicated in the reduction of vascular smooth muscle tone and arteriolar vasodilation (Branco et al., 2014). Both of these actions can result in an increase in the blood flow to the skin, delivering heat to the body surface that is dissipated into the surrounding environment (Branco et al., 2014). It is hypothesized that NO-mediated responses to increasing dietary Arg resulted in the dose-dependent core body temperature responses that we observed during the hot period in experiment 1. Although we found no statistical significance in plasma total NO concentrations at either 38 or 46 d in either of our experiments, the effects of NO on body temperature are mostly attributed to the constitutive expression of endothelial (eNOS) and neuronal (nNOS) nitric oxide synthase (**NOS**) enzymes. These enzymes produce NO at smaller concentrations than those observed with the inducible NOS isoform (Gerstberger, 1999; Coleman, 2001; Chapman and Wideman, 2006; Hirst and Robson, 2011), and it is known that biologically effective levels of NO produced in low concentrations can be undetectable by colorimetric assays for total plasma NO (Chapman and Wideman, 2006). A study by Uyanga et al. (2022) reported that intracerebroventricular treatment of 4 μg L-Cit reduced the body surface temperature as well as rectal temperature of broilers, but it did not influence the total plasma NO. Instead, hypothalamic concentrations of NO tended to increase.

In contrast to the results of experiment 1, dietary Arg did not influence the cloacal temperatures measured in experiment 2 at any sampling point. An obvious suggestion would be that body temperature responses to dietary Arg may be influenced by broiler genotype. However, it is worth noting that the average 46 d concentration of plasma NO in experiment 2 was nearly double that of experiment 1, and higher than that typically reported in the literature for healthy

birds (Allen and Fetterer, 2000; Rochell et al., 2017; Anderson, Chapter 3). Paired with high mortality of this genotype (13.5% from 27 to 46 d), these data may suggest an inflammatory status of the animal with activation of iNOS (Allen and Fetterer, 2000; Coleman, 2001), overwhelming the potential for dietary Arg to influence body temperature regulation through constitutive NOS isoforms. A study by Brugaletta et al. (2023a) also reported that Arg supplementation did not influence hyperthermia in Ross 308 broilers during HS, but only two Arg concentrations were utilized in this study and thus linear and quadratic trends with increasing doses of Arg could not be evaluated. Further research investigating the influence of dietary Arg on cloacal body temperatures of different broiler genotypes, HS models, and inflammatory and health statuses is warranted.

In mild or moderate stress conditions in healthy birds, the number of heterophils increases (Maxwell and Robertson, 1998), leading to the use of the heterophil to lymphocyte (**H:L**) ratio as a reliable and accurate indicator of the physiological stress response in chickens (Maxwell and Robertson, 1998). Previous work has shown that constant HS can increase the blood concentration of heterophils and possibly decrease that of lymphocytes leading to a higher H:L ratio (Awaad et al., 2018; Olfati et al., 2018), although data for cyclic HS is less consistent (Xu et al., 2018b; Wang et al., 2018; Rocchi et al., 2022; Rocchi et al., 2023). Regardless, there is evidence to suggest that dietary Arg may also influence these leukocyte populations and thus the H:L ratio in poultry (Lee et al., 2002; Jahanian, 2009; Cengizi and Küçükersan, 2010; Al-Daraji and Salih, 2012; Oso et al., 2017). In our study, however, neither the concentrations or proportions of heterophils, lymphocytes, or the H:L ratio measured at 46 d were significantly influenced by dietary Arg ratio, and thus the results of our experiments do not support these data at least in our broilers that were chronically heat-stressed.

Plasma AGP concentration measured in experiment 1 was also not influenced by dietary dArg:dLys ratio at either 38 or 46 d, or in experiment 2 at 46 d. Work that evaluates the effect of Arg on AGP in chickens is not extensively reported, but some studies utilizing lipopolysaccharide (**LPS**) injections to initiate the inflammatory response show that plasma AGP concentration can increase with increasing dietary Arg concentration (Takahashi et al., 1999; Anderson, Chapter 3). However, these responses were thought to coincide with production of NO (from iNOS activation), and thus it may not be surprising that plasma AGP concentration in our study did not differ as HS is less inflammatory than LPS. This idea is consistent with work by Dao et al. (2022), who used a subclinical model of necrotic enteritis and found no differences in serum AGP or the jejunal relative mRNA expression of NOS2 in challenged broilers fed reduced CP diets with or without supplemental Arg. Although, it is interesting to note that Takahashi et al. (1999) reported chicks fed an Arg deficient diet had higher plasma AGP level prior to the LPS injection compared to those fed an Arg sufficient diet.

Due to logistical constraints, plasma concentrations of AA were only measured in experiment 1. Our data show that the plasma AA pool was certainly affected by increasing the dArg:dLys ratio under HS at both 38 and 46 d time points. It is well known that HS can alter protein synthesis and degradation in broilers (Teyssier et al., 2023), and thus, changes in the plasma free AA pool can be expected (Tabiri et al., 2000; Jafari et al., 2020; Ma et al., 2021). However, it was not known how dietary Arg concentration may affect the broiler plasma AA pool during chronic HS. In our experiment, increasing the dArg:dLys ratio resulted in a linear increase in plasma Arg concentration across both time points, confirming that as dietary Arg increases so does the amount of Arg that is bioavailable to the animal for metabolic use. This linear increase occurred despite the fact that HS has been shown to decrease the ileal digestibility

of Arg by some studies (Balnave and Oliva, 1991; Soleimani et al., 2010), although not others (Teyssier et al., 2023). Although, it should also be taken into account that the effect of HS on digestibility may be tied to source, as free AA are more digestible and less likely to have this factor be impacted by HS (Morales et al., 2016).

Ornithine and Cit are products of Arg catabolism by arginase and NOS enzymes, respectively (Khajali and Wideman, 2010). Along with plasma Arg, concentrations of these AA also linearly increased at both time points as dietary Arg increased. Ornithine is a precursor for the synthesis of polyamines, which have several important functions in cell growth, proliferation, and differentiation (Pegg, 2016; Furukawa et al., 2021), and Cit is a by-product of NO synthesis (Khajali and Wideman, 2010). It is not known whether these AA were synthesized from Arg with biological purpose with more available substrate, or if they rather serve as a reflection of an effort to eliminate excess Arg. Proline (**Pro**) is also synthesized from Orn, and is a major component of collagen beneficial for wound healing and tissue remodeling (Morris, 2007; Khajali and Wideman, 2010). Interestingly, plasma concentrations of Pro linearly decreased at 38 d as dietary Arg increased, and tended to decrease ($P = 0.070$) at 46 d.

Other AA consistently affected by dietary dArg:dLys ratio include Ser, Gln, Gly, Thr, and alanine (**Ala**). The concentration of each of these AA decreased linearly as Arg in the diet increased. Changes in the plasma levels of these AA may be attributed to changes in N metabolism, as additional dietary Arg may affect N balance in the animal. Several of these AA (particularly Gln and Ala) can be used as a reservoir for excess N (Wu, 2021), and it has been suggested that a decreased concentration of plasma Gly may occur with an increased demand for uric acid or creatine synthesis (Maruyama et al., 1976; Kidd and Kerr, 1996). In fact, carbon skeletons from the catabolism of Thr can generate Gly (Kidd and Kerr, 1996). Alternatively, it

has also been suggested that reductions in the concentrations of some of the gluconeogenic amino acids in chickens may indicate gluconeogenesis under HS (Tabiri et al., 2000).

Increasing the dArg:dLys ratio during the finisher phase in our study also had effects on the 48 d processing characteristics of both broiler genotypes reared under cyclic HS. As both cyclic and constant HS models can cause significant reductions in hot and chilled carcass weight as well as breast meat weight and yield among other carcass changes (Zeferino et al., 2016; Teyssier et al., 2022a; Cartoni Mancinelli et al., 2023), nutritional interventions that help mitigate some of these negative effects may be particularly valuable. It is interesting to note that the changes in processing traits in our study were not consistent across experiments, again indicating that broiler responsiveness to dietary Arg may vary. Experiment 1 showed a quadratic and linear increase in chilled carcass weight and yield, respectively, with carcass yield increasing 0.95% from lowest to highest dArg:dLys ratio, mostly as a result of the increase in TBM. Individually, breast fillet and tender weight also both significantly increased. Previous studies demonstrate that increased dietary Arg can have similar effects on the carcass traits of broilers reared under thermoneutral conditions (Ebrahimi et al., 2014; Pirsaraei et al., 2018; Sirathonpong et al., 2019; Westreicher-Kristen et al., 2025); however, not always under HS (Nassar et al., 2022; Abd El-Maged and Desoky, 2023; Oliveira et al., 2024).

In experiment 2, we observed a linear increase in the absolute weight of the thigh and drum with increasing dietary dArg:dLys ratio, although there was no significant difference in the weight of the total chilled carcass. There was also a quadratic decrease in fat pad weight and yield. Because HS models have shown to cause an increase in the relative weight of abdominal fat (Zeferino et al., 2016; Teyssier et al., 2022a), an Arg-mediated decrease in fat pad yield may be notable. Lower fat pad yield with Arg supplementation was also reported by Nassar et al.

(2022) when broilers were exposed to cyclic HS, and it has been hypothesized that the Arg effect on lipid metabolism may be due to a NO-mediated alteration of lipogenic gene expression (Ebrahimi et al., 2014; Pirsaraei et al., 2018; Oliveira et al., 2024).

Overall, increasing the dietary dArg:dLys ratio from 0.80 to 1.40 for broilers experiencing cyclic HS led to dose-dependent patterns of improvement in FCR, cloacal body temperature, and 48 d processing weights and yields. These results indicate that feeding Arg ratios higher than current recommendations may be beneficial for improving broiler performance and health. However, it is important to note that the responses to dietary Arg varied based on genotype, which is further supported by the fact the experiments in our study were run concurrently and thus identical in other variables. This highlights the sensitivity of genotype as an influential factor in the variability of results, both in this experiment and in general.

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Table 4.1. Ingredient and calculated nutrient composition of common starter and grower diets – Experiments 1 and 2 (% as-fed, unless otherwise noted).

	Starter		Grower	
Item	Exp. 1 – Ross (0 to 17 d)	Exp. 2 – Cobb (0 to 14 d)	Exp. 1 – Ross (17 to 27 d)	Exp. 2 – Cobb (14 to 27 d)
Corn	60.280	59.107	65.736	64.859
Soybean meal	29.234	30.332	22.429	22.388
DDGS[1]	2.500	2.500	2.500	2.500
Wheat middlings	0.343	1.750	1.000	2.000
Poultry by-product meal	3.000	1.355	3.000	3.000
Corn gluten meal	1.250	1.250	1.500	1.500
Fat, animal	0.500	0.650	0.860	0.750
Calcium carbonate	0.816	0.891	0.842	0.900
Dicalcium phosphate	0.880	0.843	0.735	0.722
Salt	0.285	0.329	0.262	0.388
Sodium sesquicarbonate	0.041	0.075	0.098	-
L-Methionine	0.249	0.248	0.283	0.248
L-Lysine·HCl	0.218	0.223	0.291	0.288
Threonine fermentate	0.124	0.131	0.089	0.088
L-Arginine	0.008	0.028	0.074	0.069
L-Isoleucine	-	-	0.001	-
Trace mineral premix[2]	0.100	0.100	0.100	0.100
Vitamin premix[3]	0.100	0.100	0.100	0.100
Coccidiostat[4]	0.050	0.050	0.050	0.050
Choline chloride	0.010	0.025	0.037	0.037
Xylanase[5]	0.008	0.008	0.008	0.008
Phytase[6]	0.005	0.005	0.005	0.005
Calculated nutrients				
ME, kcal/kg	3,020	2,998	3,089	3,067
CP	22.34	21.94	20.58	20.62
Digestible Lys	1.230	1.220	1.100	1.100
Digestible Arg	1.304	1.293	1.166	1.166

[1]Distillers dried grains with solubles.
[2]Mineral premix includes per kg of diet: Mn (manganese sulfate), 120 mg; Zn (zinc sulfate), 100 mg; Fe (ferrous sulfate), 30 mg; Cu (basic copper chloride), 8 mg; I (stabilized ethylenediamine dihydroiodide), 1.4 mg; Se (sodium selenite), 0.3 mg.
[3]Vitamin premix includes per kg of diet: Vitamin A, 18,739 IU; Vitamin D3, 6,614 IU; Vitamin E, 66 IU; niacin, 88 mg; d-pantothenic acid (d-calcium pantothenate), 31 mg; riboflavin, 22 mg; Vitamin B6 (pyridoxine hydrochloride), 7.7 mg; thiamine (thiamine mononitrate), 5.5 mg; menadione (menadione sodium bisulfite complex), 4 mg; folic acid, 2.6 mg; biotin, 0.18 mg; Vitamin B12, 0.03 mg.
[4]Zoamix® provides 250 g of zoalene per kg of premix (Zoetis, Parsippany, NJ).
[5]Econase XT (AB Vista Feed Ingredients, Marlborough, UK).
[6]Quantum Blue 10G (AB Vista Feed Ingredients, Marlborough, UK).

Table 4.2. Ingredient, calculated, and analyzed nutrient composition of the experimental finisher 1 and finisher 2 Diet A – Experiments 1 and 2 (% as-fed, unless otherwise noted).[1]

Item	Finisher 1 – 27 to 38 d	Finisher 2 – 38 to 46 d
Corn	69.902	73.362
Soybean meal	13.772	12.347
DDGS[2]	7.499	5.734
Corn gluten meal	4.000	3.250
Fat, animal	0.688	1.278
Calcium carbonate	1.016	1.011
Dicalcium phosphate	0.628	0.679
Salt	0.244	0.261
Potassium carbonate	0.167	0.181
Sodium sesquicarbonate	0.091	0.119
Inert filler (sand)	0.609	0.560
L-Lysine·HCl	0.490	0.453
L-Methionine	0.270	0.242
Threonine fermentate	0.157	0.145
L-Isoleucine	0.088	0.084
L-Valine	0.074	0.069
L-Tryptophan	0.030	0.028
Trace mineral premix[3]	0.100	0.100
Vitamin premix[4]	0.100	0.050
Coccidiostat[5]	0.050	-
Choline chloride	0.012	0.035
Xylanase[6]	0.008	0.008
Phytase[7]	0.005	0.005
Calculated nutrients		
ME, kcal/kg	3,109	3,164
CP	17.95	16.50
Digestible Lys	1.000	0.920
Digestible Arg	0.805	0.741
Analyzed nutrients[8,9]		
CP	18.80	16.71
Total Lys	1.16	1.08
Total Arg	0.98	0.91

[1]Crystalline L-Arginine was added to Diet A (calculated dArg:dLys ratio of 0.80) at the expense of inert filler in order to meet calculated dArg:dLys ratios for Diets B-F (0.92, 1.04, 1.16, 1.28, and 1.40, respectively).

[2]Distillers dried grains with solubles.

[3]Mineral premix includes per kg of diet: Mn (manganese sulfate), 120 mg; Zn (zinc sulfate), 100 mg; Fe (ferrous sulfate), 30 mg; Cu (basic copper chloride), 8 mg; I (stabilized ethylenediamine dihydroiodide), 1.4 mg; Se (sodium selenite), 0.3 mg.

[4]Vitamin premix at 0.100% inclusion rate includes per kg of diet: Vitamin A, 18,739 IU; Vitamin D3, 6,614 IU; Vitamin E, 66 IU; niacin, 88 mg; d-pantothenic acid (d-calcium pantothenate), 31 mg; riboflavin, 22 mg; Vitamin B6 (pyridoxine hydrochloride), 7.7 mg; thiamine (thiamine

mononitrate), 5.5 mg; menadione (menadione sodium bisulfite complex), 4 mg; folic acid, 2.6 mg; biotin, 0.18 mg; Vitamin B12, 0.03 mg.
[5]Zoamix® provides 250 g of zoalene per kg of premix (Zoetis, Parsippany, NJ).
[6]Econase XT (AB Vista Feed Ingredients, Marlborough, UK).
[7]Quantum Blue 10G (AB Vista Feed Ingredients, Marlborough, UK).
[8]The analyzed total Arg:Lys ratios for experimental finisher 1 diets B-F was 0.84, 0.92, 1.00, 1.09, 1.21, and 1.29, respectively. The analyzed total Arg:Lys ratios for experimental finisher 2 diets B-F was 0.84, 0.93, 1.02, 1.11, 1.19, and 1.30, respectively.
[9]The analyzed free Arg concentration for experimental finisher 1 diets A-F was 0.03, 0.13, 0.24, 0.36, 0.46, and 0.55%, respectively. The analyzed free Arg concentration for experimental finisher 2 diets A-F was 0.4, 0.12, 0.22, 0.32, 0.42, and 0.50%, respectively.

Table 4.3. Growth performance of male broilers fed increasing concentrations of Arg from 27 to 46 d post-hatch – Experiments 1 and 2[1].

	Arg Ratio							*P*-values		
Item	0.80	0.92	1.04	1.16	1.28	1.40	SEM	ANOVA	Linear	Quadratic
Experiment 1 – Ross 708[2]										
27 to 38 d: Finisher 1 Phase										
27 d BW, kg	1.473	1.486	1.483	1.480	1.483	1.485	0.009	0.892	0.499	0.692
38 d BW, kg	2.388	2.405	2.441	2.424	2.436	2.426	0.017	0.243	0.066	0.145
BWG, kg	0.916	0.920	0.958	0.946	0.954	0.941	0.017	0.411	0.139	0.204
FI, kg	1.698	1.698	1.701	1.689	1.676	1.703	0.017	0.890	0.723	0.676
FCR, kg:kg	1.872[a]	1.859[ab]	1.789[ab]	1.799[ab]	1.763[b]	1.809[ab]	0.025	***0.027***	***0.001***	0.068
27 to 46 d: Cumulative Finisher Phase										
46 d BW, kg	2.904	2.929	3.019	2.974	2.955	2.983	0.030	0.131	0.099	0.136
BWG, kg	1.429	1.443	1.535	1.495	1.471	1.499	0.030	0.149	0.120	0.155
FI, kg	2.874	2.889	2.938	2.905	2.879	2.910	0.034	0.796	0.682	0.492
FCR, kg:kg	2.045	2.035	1.934	1.981	1.964	1.957	0.028	***0.039***[3]	***0.012***	0.176
Experiment 2 – Cobb 500[4]										
27 to 38 d: Finisher 1 Phase										
27 d BW, kg	1.711	1.710	1.713	1.720	1.714	1.716	0.011	0.989	0.631	0.870
38 d BW, kg	2.633	2.625	2.611	2.678	2.685	2.668	0.033	0.509	0.134	0.908
BWG, kg	0.919	0.914	0.899	0.958	0.971	0.953	0.031	0.499	0.126	0.870
FI, kg	1.831	1.796	1.766	1.808	1.791	1.818	0.023	0.429	0.823	0.088
FCR, kg:kg	2.067	1.988	1.989	1.943	1.890	1.957	0.043	0.122	***0.018***	0.202
27 to 46 d: Cumulative Finisher Phase										
46 d BW, kg	3.096	3.144	3.103	3.189	3.170	3.163	0.062	0.867	0.342	0.745
BWG, kg	1.385	1.434	1.390	1.468	1.455	1.446	0.059	0.884	0.369	0.765
FI, kg	3.026	2.993	2.960	3.035	2.999	3.048	0.052	0.855	0.648	0.409
FCR, kg:kg	2.326	2.248	2.197	2.148	2.127	2.180	0.069	0.371	0.056	0.225

[a-b]Means within a row that do not share a common superscript were deemed different ($P \leq 0.05$) by a Tukey's multiple comparison test.

[1]A cyclic heat stress model was used from 28 to 46 d. Environmental temperature was maintained at 32°C for 12 h daily (07:30 to 19:30) and reduced to 24°C each night.

[2]Values are least square means of 8 replicate pens per treatment with 25 birds per pen.

[3]A Tukey's multiple comparison test did not deem differences among treatments.

[4]Values are least square means of 8 replicate pens per treatment with 23 birds per pen.

Table 4.4. Cloacal temperatures (°C) of male broilers fed increasing concentrations of Arg, measured at 32, 39, and 46 d post-hatch – Experiments 1 and 2[1,2].

	Arg Ratio							*P*-values		
Item	0.80	0.92	1.04	1.16	1.28	1.40	SEM	ANOVA	Linear	Quadratic
Experiment 1 – Ross 708										
32 d										
06:00	41.21	41.13	41.17	41.13	40.99	41.16	0.094	0.681	0.366	0.527
14:00	43.15	43.06	42.95	42.66	42.96	43.25	0.162	0.187	0.949	***0.022***
Difference	1.94	1.93	1.78	1.54	1.96	2.09	0.180	0.356	0.678	0.078
39 d										
06:00	41.66	41.70	41.64	41.57	41.33	41.66	0.104	0.157	0.172	0.443
14:00	43.34	43.28	43.14	42.93	42.99	43.39	0.139	0.131	0.486	***0.021***
Difference	1.68	1.58	1.50	1.36	1.66	1.73	0.135	0.420	0.733	0.065
46 d										
06:00	42.05	41.87	41.84	41.79	41.78	41.81	0.100	0.419	0.076	0.220
14:00	43.48[a]	43.39[ab]	43.30[ab]	43.06[ab]	42.88[b]	43.27[ab]	0.124	***0.023***	***0.011***	0.087
Difference	1.43	1.52	1.46	1.28	1.33	1.46	0.159	0.892	0.651	0.664
Experiment 2 – Cobb 500										
32 d										
06:00	41.19	41.19	41.19	41.19	41.23	41.23	0.102	0.999	0.720	0.894
14:00	42.79	42.81	42.96	42.93	43.09	43.04	0.139	0.576	0.081	0.822
Difference	1.61	1.62	1.78	1.74	1.87	1.82	0.173	0.861	0.227	0.796
39 d										
06:00	41.68	41.59	41.79	41.76	41.82	41.81	0.109	0.665	0.168	0.861
14:00	43.13	43.14	43.14	43.33	43.23	43.30	0.129	0.812	0.235	0.942
Difference	1.45	1.54	1.36	1.56	1.41	1.49	0.113	0.782	0.974	0.933
46 d										
06:00	41.99	41.77	41.89	42.05	41.79	41.89	0.091	0.224	0.731	0.881
14:00	43.54	43.31	43.58	43.34	43.76	43.53	0.140	0.240	0.365	0.646
Difference	1.54	1.54	1.69	1.29	1.97	1.64	0.145	0.057	0.278	0.727

[a-b]Means within a row that do not share a common superscript were deemed different ($P \leq 0.05$) by a Tukey's multiple comparison test.

[1]Values are least square means of 8 replicate pens per treatment of 2 birds per pen. At each time point, data were collected at 06:00 (before daily heat was applied) and at 14:00 (during peak daily heat application).

[2]A cyclic heat stress model was used from 28 to 46 d. Environmental temperature was maintained at 32°C for 12 h daily (07:30 to 19:30) and reduced to 24°C each night.

Table 4.5. Heterophil and lymphocyte counts of male broilers at 46 d post-hatch fed increasing concentrations of Arg – Experiments 1 and 2.[1,2]

		Arg Ratio							*P*-values		
Item		0.80	0.92	1.04	1.16	1.28	1.40	SEM	ANOVA	Linear	Quadratic
Experiment 1 – Ross 708											
Heterophils	10^3/uL	3.652	4.272	4.022	3.472	3.285	3.600	0.380	0.340	0.176	0.664
	%	56.29	60.71	59.14	57.05	50.29	57.71	3.816	0.415	0.397	0.866
Lymphocytes	10^3/uL	2.646	2.892	2.432	2.523	2.850	2.255	0.320	0.542	0.402	0.430
	%	38.86	35.57	35.86	39.81	43.86	36.57	3.446	0.449	0.534	0.871
H/L		1.500	1.999	1.713	1.506	1.243	1.666	0.254	0.352	0.423	0.899
Experiment 2 – Cobb 500											
Heterophils	10^3/uL	4.340	4.167	4.205	4.341	5.673	3.761	0.605	0.287	0.721	0.515
	%	58.71	51.82	57.49	53.00	59.71	60.24	4.108	0.470	0.399	0.237
Lymphocytes	10^3/uL	2.662	3.282	2.716	3.192	3.211	2.762	0.366	0.659	0.797	0.360
	%	35.71	42.83	37.67	40.14	34.43	39.00	3.380	0.504	0.820	0.621
H/L		1.732	1.267	1.607	1.436	1.822	1.525	0.223	0.510	0.804	0.610

[1]Values are least square means of 7 replicate pens per treatment of 1 bird per pen.

[2]A cyclic heat stress model was used from 28 to 47 d. Environmental temperature was maintained at 32°C for 12 h daily (07:30 to 19:30) and reduced to 24°C each night.

Table 4.6. Plasma metabolite concentrations of Ross 708 male broilers at 38 d post-hatch fed increasing concentrations of Arg – Experiment 1.[1,2]

	Arg Ratio							*P*-values		
Item	0.80	0.92	1.04	1.16	1.28	1.40	SEM	ANOVA	Linear	Quadratic
Blood Metabolites										
NO[3]	13.055	16.046	14.895	15.358	13.702	13.723	1.087	0.369	0.725	0.099
AGP[4]	0.327	0.287	0.318	0.289	0.265	0.249	0.033	0.540	0.088	0.743
Essential Amino Acids (μM/L)										
Arg	84[d]	160[cd]	182[bc]	264[ab]	295[a]	317[a]	21.43	***<0.0001***	***<0.0001***	0.240
His	27	24	23	26	23	28	2.93	0.762	0.986	0.294
Ile	35	33	31	36	31	26	3.25	0.319	0.092	0.341
Leu	108	98	93	104	97	92	5.22	0.273	0.113	0.708
Lys	179	131	117	159	99	128	27.22	0.313	0.163	0.398
Met	59	53	55	59	60	51	3.89	0.536	0.687	0.661
Phe	67	65	64	65	62	62	3.06	0.864	0.236	0.950
Thr	411[a]	309[b]	292[b]	333[ab]	268[b]	259[b]	21.69	***<0.0001***	***<0.0001***	0.172
Val	83	77	69	78	69	71	5.94	0.450	0.119	0.534
Nonessential Amino Acids (μM/L)										
Ala	329[a]	260[ab]	235[b]	250[ab]	279[ab]	214[b]	20.61	***0.010***	***0.007***	0.224
Asn	82	77	69	70	72	56	9.63	0.545	0.082	0.864
Asp	39	30	30	27	27	31	4.56	0.480	0.189	0.127
Cit	5.9	5.8	7.2	8.0	8.5	8.2	0.653	***0.014***[5]	***0.001***	0.388
Gln	724[a]	592[ab]	535[b]	526[b]	508[b]	494[b]	36.21	***0.001***	***<0.0001***	***0.023***
Glu	74	71	64	73	65	66	4.43	0.429	0.160	0.656
Gly	385[a]	341[ab]	303[ab]	302[ab]	295[b]	284[b]	20.68	***0.015***	***0.001***	0.138
Orn	9.3[c]	10.0[c]	11.2[bc]	24.8[a]	23.1[ab]	23.6[ab]	3.289	***0.001***	***<0.0001***	0.669
Pro	318[a]	266[ab]	269[ab]	265[ab]	261[ab]	230[b]	16.58	***0.027***	***0.002***	0.592
Ser	323[a]	292[ab]	274[ab]	272[ab]	286[ab]	242[b]	14.75	***0.015***	***0.002***	0.648
Tau	44	53	39	48	49	33	5.91	0.238	0.239	0.260
Tyr	136	112	110	107	137	99	11.80	0.135	0.266	0.606

[a-d]Means within a row that do not share a common superscript were deemed different ($P \leq 0.05$) by a Tukey's multiple comparison test.

[1]Values are least square means of 8 replicate pens per treatment of 1 bird per pen.

[2]A cyclic heat stress model was used from 28 to 47 d. Environmental temperature was maintained at 32°C for 12 h daily (07:30 to 19:30) and reduced to 24°C each night.
[3]Nitric oxide; µM.
[4]Alpha-1-acid glycoprotein; mg/mL.
[5]A Tukey's multiple comparison test did not deem differences among treatments.

Table 4.7. Plasma metabolite concentrations of Ross 708 male broilers at 46 d post-hatch fed increasing concentrations of Arg – Experiment 1.[1,2]

	Arg Ratio							*P*-values		
Item	0.80	0.92	1.04	1.16	1.28	1.40	SEM	ANOVA	Linear	Quadratic
Blood Metabolites										
NO^3	13.558	16.059	9.820	12.073	12.650	11.799	2.494	0.633	0.427	0.649
AGP[4]	0.346	0.514	0.249	0.374	0.273	0.290	0.098	0.361	0.268	0.907
Essential amino acids (μM/L)										
Arg	96[b]	163[ab]	199[a]	210[a]	222[a]	254[a]	22.83	***<0.0001***	***<0.0001***	0.204
His	32[ab]	31[ab]	34[ab]	27[ab]	23[b]	35[a]	2.68	***0.034***	0.454	0.116
Ile	41	31	33	31	24	33	4.89	0.305	0.133	0.199
Leu	101	92	106	95	95	93	4.36	0.153	0.221	0.609
Lys	138	141	144	146	81	142	25.40	0.348	0.456	0.934
Met	50	40	48	49	46	42	3.21	0.276	0.478	0.665
Phe	78[ab]	77[ab]	82[a]	78[ab]	70[b]	71[ab]	2.74	***0.028***	***0.009***	0.170
Thr	297[a]	235[ab]	220[ab]	210[b]	183[b]	186[b]	19.81	***0.001***	***<0.0001***	0.115
Val	85[a]	76[ab]	86[a]	73[ab]	63[b]	74[ab]	4.59	***0.015***	***0.009***	0.622
Nonessential amino acids (μM/L)										
Ala	248[a]	179[b]	181[b]	212[ab]	214[ab]	171[b]	15.70	***0.011***	0.066	0.357
Asn	66[a]	54[ab]	52[ab]	55[ab]	44[b]	39[b]	5.32	***0.012***	***<0.0001***	0.997
Asp	32	28	34	31	37	34	4.00	0.749	0.332	0.926
Cit	5.0	5.0	6.9	8.2	7.8	6.4	0.789	***0.023***[5]	***0.015***	***0.033***
Gln	600	516	509	492	446	468	36.27	0.082	***0.006***	0.269
Glu	207	201	213	212	204	175	17.56	0.679	0.305	0.242
Gly	360	335	318	358	297	287	20.98	0.058	***0.013***	0.566
Orn	10.8[c]	20.3[bc]	24.8[bc]	35.7[ab]	27.0[bc]	47.6[a]	4.665	***<0.0001***	***<0.0001***	0.943
Pro	189	171	178	180	163	154	12.67	0.441	0.070	0.648
Ser	335	294	302	296	301	267	16.35	0.143	***0.022***	0.895
Tau	42	44	40	42	50	33	5.32	0.373	0.596	0.313
Tyr	102	82	87	112	114	78	9.34	***0.034***[5]	0.991	0.316

[a-c]Means within a row that do not share a common superscript were deemed different ($P \leq 0.05$) by a Tukey's multiple comparison test.

[1]Values are least square means of 7 replicate pens per treatment of 1 bird per pen.

[2]A cyclic heat stress model was used from 28 to 47 d. Environmental temperature was maintained at 32°C for 12 h daily (07:30 to 19:30) and reduced to 24°C each night.

[3]Nitric oxide; µM.
[4]Alpha-1-acid glycoprotein; mg/mL.
[5]A Tukey's multiple comparison test did not deem differences among treatments.

Table 4.8. Processing characteristics of Ross 708 male broilers at 48 d post-hatch fed increasing concentrations of Arg – Experiment 1.[1,2]

		Arg Ratio							*P*-values		
Item		0.80	0.92	1.04	1.16	1.28	1.40	SEM	ANOVA	Linear	Quadratic
Live[3]	kg	2.999	3.030	3.140	3.094	3.119	3.064	0.043	0.192	0.136	0.057
Chilled	kg	2.318	2.363	2.437	2.418	2.437	2.397	0.032	0.081	***0.032***	***0.034***
Carcass	%	77.29	77.97	77.56	78.18	78.14	78.24	0.244	***0.044***[4]	***0.006***	0.524
Fat Pad	kg	0.032	0.034	0.034	0.036	0.034	0.037	0.002	0.376	0.064	0.826
	%	1.08	1.10	1.08	1.15	1.08	1.20	0.048	0.377	0.135	0.505
Breast	kg	0.639	0.657	0.682	0.684	0.687	0.654	0.013	0.067	0.140	***0.007***
Fillets	%	21.29	21.64	21.67	22.05	21.97	21.32	0.243	0.150	0.447	***0.018***
Tenders	kg	0.139	0.145	0.148	0.149	0.150	0.147	0.003	0.063	***0.013***	***0.040***
	%	4.64	4.78	4.71	4.82	4.82	4.80	0.059	0.199	***0.038***	0.387
Total	kg	0.778	0.802	0.830	0.831	0.837	0.801	0.015	0.067	0.094	***0.009***
Breast	%	25.92	26.42	26.37	26.82	26.79	26.12	0.265	0.132	0.252	***0.023***
Wings	kg	0.252	0.253	0.261	0.255	0.258	0.257	0.003	0.254	0.188	0.268
	%	8.41	8.35	8.33	8.25	8.27	8.39	0.067	0.477	0.453	0.089
Thighs	kg	0.390	0.393	0.406	0.401	0.407	0.404	0.007	0.321	0.057	0.323
	%	12.99	12.95	12.94	13.00	13.06	13.18	0.144	0.844	0.273	0.387
Drums	kg	0.308	0.312	0.317	0.312	0.313	0.314	0.004	0.789	0.442	0.447
	%	10.26	10.20	10.09	10.08	10.05	10.24	0.077	0.253	0.401	***0.031***

[1]Values are least square means of 8 replicate pens per treatment of 8 birds per pen. Yield was calculated as a percentage of live weight.
[2]A cyclic heat stress model was used from 28 to 47 d. Environmental temperature was maintained at 32°C for 12 h daily (07:30 to 19:30) and reduced to 24°C each night.
[3]Individual live weights taken the afternoon before the day of processing (47 d).
[4]A Tukey's multiple comparison test did not deem differences among treatments.

Table 4.9. Processing characteristics of Cobb 500 male broilers at 48 d post-hatch fed increasing concentrations of Arg – Experiment 2.[1,2]

		Arg Ratio							*P*-values		
Item		0.80	0.92	1.04	1.16	1.28	1.40	SEM	ANOVA	Linear	Quadratic
Live[3]	kg	3.215	3.206	3.220	3.193	3.271	3.337	0.068	0.678	0.180	0.323
Chilled	kg	2.461	2.467	2.477	2.455	2.503	2.571	0.048	0.535	0.118	0.302
Carcass	%	76.60	76.97	77.02	76.83	76.57	77.06	0.043	0.942	0.805	0.877
Fat Pad	kg	0.039[a]	0.033[b]	0.036[ab]	0.036[ab]	0.035[ab]	0.039[a]	0.001	***0.012***	0.836	***0.005***
	%	1.22[a]	1.03[b]	1.11[ab]	1.11[ab]	1.05[b]	1.14[ab]	0.039	***0.021***	0.338	***0.019***
Breast	kg	0.642	0.666	0.646	0.649	0.666	0.679	0.018	0.647	0.214	0.555
Fillets	%	19.86	20.70	20.02	20.21	20.31	20.28	0.214	0.133	0.653	0.529
Tenders	kg	0.137	0.135	0.136	0.137	0.140	0.139	0.003	0.941	0.389	0.760
	%	4.23	4.21	4.24	4.29	4.26	4.15	0.076	0.840	0.767	0.336
Total	kg	0.779	0.801	0.783	0.786	0.805	0.818	0.020	0.720	0.219	0.571
Breast	%	24.09	24.91	24.26	24.50	24.58	24.43	0.251	0.301	0.654	0.408
Wings	kg	0.274	0.272	0.277	0.274	0.272	0.281	0.005	0.801	0.497	0.591
	%	8.51	8.51	8.64	8.58	8.34	8.43	0.088	0.212	0.186	0.217
Thighs	kg	0.431	0.427	0.441	0.438	0.445	0.455	0.007	0.131	***0.009***	0.535
	%	13.43	13.37	13.76	13.40	13.64	13.65	0.171	0.497	0.286	0.908
Drums	kg	0.332	0.330	0.339	0.330	0.343	0.348	0.006	0.179	***0.035***	0.320
	%	10.34	10.32	10.54	10.32	10.49	10.46	0.090	0.343	0.240	0.804

[a-b]Means within a row that do not share a common superscript were deemed different ($P \leq 0.05$) by a Tukey's multiple comparison test.

[1]Values are least square means of 8 replicate pens per treatment of 8 birds per pen. Yield was calculated as a percentage of live weight.

[2]A cyclic heat stress model was used from 28 to 47 d. Environmental temperature was maintained at 32°C for 12 h daily (07:30 to 19:30) and reduced to 24°C each night.

[3]Individual live weights taken the afternoon before the day of processing (47 d).

V. DIETARY ARGININE RESPONSES OF ROSS 708 BROILERS SUBJECTED TO ENTERIC CHALLENGE WITH *EIMERIA* SPP. AND *CLOSTRIDIUM PERFRINGENS*

ABSTRACT

The objective of this study was to evaluate the effects of dietary Arg ratio on performance and processing characteristics of broilers subjected to an enteric challenge with *Eimeria* spp. and *Clostridium perfringens*. A total of 1,800 male Ross 708 broiler chicks were fed a common starter diet (0 to 11 d) prior to being fed 1 of 7 target digestible Arg:Lys ratios (0.81, 0.93, 1.05, 1.17, 1.29, 1.41, and 1.53) from 11 to 29 d and a common finisher diet (29 to 39 d). At 15 d, birds were orally gavaged with a 10-fold dose of a live coccidiosis vaccine followed by ~10^7 CFU *C. perfringens* at 19 and 20 d. Unchallenged birds in control groups (1.05 and 1.29 dArg:dLys only) received a sham gavage. At 39 d, 12 birds per pen from 4 treatments (1.05 or 1.29 dArg:dLys × unchallenged or challenged) were processed and deboned to determine carcass and parts weights and yields. Data were analyzed by ANOVA with pen location as a random blocking variable, and pre-planned contrasts were used to evaluate the main effects of dArg:dLys ratio (1.05 or 1.29 dArg:dLys), enteric challenge, and their interaction. Polynomial contrasts were also used to determine Arg responses across all titration levels within the challenged group. Statistical significance was determined at $P \leq 0.05$. The challenge model significantly impaired growth performance during all post-challenge periods, increased plasma nitric oxide and alpha-1-acid glycoprotein concentration, and decreased plasma carotenoids and ileal secretory IgA secretion. Feeding 1.29 dArg:dLys improved feed conversion ratio (**FCR**) compared to those fed 1.05 during the challenge recovery period (22 to 29 d), and FCR of challenged birds in this phase improved linearly with increasing dArg:dLys. Furthermore,

increasing dArg:dLys ratio quadratically improved body weight gain and FCR of challenged birds during the grower phase and cumulatively, and influenced plasma concentration of amino acids. For processing characteristics, feeding 1.29 dArg:dLys improved chilled carcass yield compared to 1.05. Overall, results of this study indicate that increased dArg:dLys can improve broiler performance during enteric challenge, possibly due to the diverse roles of Arg in supporting gastrointestinal tract health and immunity.

INTRODUCTION

Enteric diseases cause significant economic losses to the broiler industry each year, with coccidiosis and necrotic enteritis (**NE**) being the two most consequential (USAHA, 2022). Coccidiosis infection caused by *Eimeria* spp. parasites results in damage to host epithelial cells, intestinal inflammation, and excess production of mucus, also making it one of the main predisposing factors for development of NE (Rodgers et al., 2015; Adhikari et al., 2020). Necrotic enteritis infection arises from an overgrowth of opportunistic *Clostridium perfringens* and results in rapid necrosis of mucosal tissue, hemorrhaging, and reduced intestinal integrity (Gholamiandehkordi et al., 2007; Van Immerseel et al., 2009; Adhikari et al., 2020). As a result, these diseases impair digestion and absorption of proteins and other nutrients that causes a mild to severe detriment to broiler growth performance, and in severe cases, high mortality rates (Van Immerseel et al., 2009; Blake and Tomley, 2014; Chalvon-Demersay et al., 2021). With increasing consumer demand for antibiotic-free poultry products, it is important to identify alternatives to subtherapeutic antibiotic growth promoters for the management of broiler responses to these challenges (Peek and Landman, 2011; Adhikari et al., 2020). Fortunately, nutritional interventions can be part of an effective strategy used to control enteric diseases in

chickens (Bortoluzzi et al., 2018; Adhikari et al., 2020; Gaignon et al., 2022; Ahmad et al., 2023).

One potential intervention is to modify the dietary inclusion of "functional" amino acids (**AA**) such as arginine (**Arg**). In addition to being a nutritionally essential AA for poultry, Arg is also a functional AA because it is a regulator of several key secondary metabolic functions that improve animal health, survival, and development beyond its use as a building block for protein synthesis and accretion (Wu, 2010; Castro and Kim, 2020). This is because Arg is the metabolic precursor to several important molecules including nitric oxide (**NO**), ornithine (**Orn**), polyamines, proline (**Pro**), glutamate, and creatine (Castro and Kim, 2020; Lee et al., 2023). Nitric oxide, for example, produced from Arg by nitric oxide synthase (**NOS**) enzymes, can be both directly cytotoxic to pathogens and act as a complex regulator of immune and inflammatory function through multiple pathways (Brunet, 2001; Coleman, 2001). In addition, polyamines, derived from Arg catabolism by arginase, have also been shown to have roles in cell division and regulation of the cell cycle (Le Floc'h et al., 2004). This translates into benefits for gastrointestinal tract development, maintenance of barrier function, and mucosal repair after injury (Löser et al., 1999; Tan et al., 2024). As a result, it has been hypothesized that the Arg needs may increase in poultry experiencing inflammation or disease stress (Kidd, 2004; Le Floc'h et al., 2004; Lee et al., 2023). Thus, the objective of this study was to evaluate the response of increasing digestible Arg ratios in broilers experiencing enteric challenge to optimize growth performance, meat yield, and recognized indicators of Arg metabolism.

MATERIALS AND METHODS

The protocol for this study was reviewed and approved by the Auburn University Institutional Animal Care and Use Committee (IACUC; animal use protocol #2024-5383).

Bird Husbandry

A total of 1,872 day-of-hatch Ross 708 male broiler chicks were obtained from a commercial hatchery (Troy, AL, USA) and delivered to the Charles C. Miller Poultry Research and Education Center at Auburn University in Auburn, AL. Chicks were placed in 72 floor pens with 26 chicks per pen, and at 11 d post-hatch, bird number was reduced to 25 birds per pen and chicks were reallotted such that all pen weights fell within 2% of the overall expected pen weight. Pens measured 2.79 m^2, which provided the minimum floor space required (0.11 m^2) for broilers up to 8 wk of age as described by The Ag Guide (Scanes et al., 2020). All pens contained fresh pine litter and were equipped with a single commercial-type pan feeder and nipple waterers to provide ad libitum access to feed and clean water throughout the trial. Supplemental waterers and TurboGrow® chick pan feeders were placed in each pen from 0 to 7 d post-hatch to facilitate access to feed and water for young chicks. Birds were monitored multiple times daily for any signs of distress (lethargy, impaired appetite, dehydration, etc.) and any birds that overtly exhibited these signs were euthanized using CO_2 inhalation or cervical dislocation. Light was provided for 23L:1D at 100% intensity during 0 to 7 d and for 18L:6D at 5-10 lux from 8 d until the end of the trial. Barn temperature was set to 32°C and decreased gradually throughout the trial to maintain bird comfort.

Experimental Diets and Design

Broilers were fed diets in three phases that included a common starter diet (0 to 11 d), an experimental grower diet (11 to 29 d), and a common finisher diet (29 to 39 d). The starter diet

was pelleted and crumbled, while the grower and finisher feeds were fed as pellets. Prior to formulation, main ingredients were analyzed for total AA profile and CP content by wet chemistry analysis. The common starter and finisher diets were corn and soybean meal-based and formulated to meet or exceed the primary breeder nutrient recommendations (Aviagen, 2022), while the experimental grower diet was formulated to be nutritionally complete and meet industry specifications except for Arg concentration. The basal grower diet was corn and soybean meal-based with inclusion of distiller's dried grains with solubles and corn gluten meal in order to achieve the lowest target Arg ratio. Crystalline L-Arg (CJ Bio, Seoul, Korea) was then added to the basal diet at the expense of an inert filler to achieve seven dietary digestible Arg:Lys ratios of 0.81, 0.93, 1.05, 1.17, 1.29, 1.41, and 1.53 (Table 5.1). All feeds were manufactured at the Auburn University feed mill. Complete feeds from each feeding phase were analyzed to determine nitrogen for CP via combustion (Method 990.03; AOAC International, 2007), total AA concentrations (Method 982.30 E [a, b, and c]; AOAC International, 2007), as well as free Arg concentration (Method 999.13; AOAC International, 2007) at the Agricultural Experiment Station Chemical Laboratories at the University of Missouri-Columbia.

The seven dArg:dLys ratios were allocated to nine treatment groups as part of two data analysis strategies described later. The full Arg titration was fed to birds subjected to enteric challenge, whereas the dArg:dLys ratio of 1.05 and 1.29 were also fed to unchallenged birds (2 × 2 factorial arrangement; enteric challenge × dArg:dLys).

Enteric Challenge Model

At 15 d post-hatch, birds were challenged by oral gavage with a 10-fold dose of a commercial live coccidiosis vaccine (ADVENT®) containing *Eimeria acervulina*, *Eimeria maxima,* and *Eimeria tenella* oocysts suspended in 0.5 mL sterilized phosphate-buffered saline

(**PBS**). At 19 and 20 d, birds were challenged by 1 mL oral gavage of 10^7 CFU and 10^6 CFU *C. perfringens*, respectively, suspended in brain heart infusion broth (Becton, Dickinson and Company). For each inoculation, *Eimeria* or *C. perfringens* were both gavaged directly into the crop using a 1 mL syringe without a needle, and unchallenged birds in control groups received a sham gavage of the respective quantities of pure medium. Fresh inoculum was prepared the morning of each challenge day. The acute phase of the challenge was considered to be from 15 to 22 d, whereas the recovery phase was considered to be from 22 to 29 d.

Growth Performance

Body weights and feed consumption were measured by pen at 0, 11, 15, 22, 29, and 39 d post-hatch to determine BW, body weight gain (**BWG**), feed intake (**FI**), and mortality-corrected FCR for each feeding phase, cumulatively, and during the period of the acute intestinal challenge and recovery. Feed intake was calculated based on number of bird days to account for mortality.

Blood and Tissue Sample Collection

At 22 d, two birds per pen were randomly selected and sampled for plasma and ileal digesta collection. Blood was collected postmortem from the femoral vessels into 4 mL BD Vacutainer™ plastic tubes spray-coated with K_2EDTA as an anticoagulant. The EDTA blood was centrifuged at 400 × *g* at 4°C for 10 min to isolate plasma. Digesta from the entire ileum was flushed with distilled water, collected, and pooled by pen for analysis of ileal secretory IgA concentration (**sIgA**). All samples were stored at -80°C until analysis.

Jejunal lesion scoring was also conducted on birds from the 2 × 2 factorial arrangement (enteric challenge × dArg:dLys) to evaluate the establishment of necrotic enteritis. Procedures were conducted by experienced personnel blinded to the experimental design following the criteria of Gholamiandehkordi et al. (2007).

Processing

At 39 d post-hatch, twelve birds from each replicate pen of the 2 × 2 factorial arrangement (enteric challenge × dArg:dLys) were selected for processing at 40 d after 11 h of feed withdrawal. Birds were individually weighed and transported to the Fortenberry Processing Plant located on site, where they were humanely euthanized, processed, and mechanically eviscerated. Carcasses were placed in ice water for approximately 3 h before cold carcass and abdominal fat pad weights were recorded. Carcasses were placed again in ice water overnight and hand-deboned the following day by professional deboners. The weights of the pectoralis major (P. major) and minor (P. minor) muscles, wings, thighs, and drums were recorded. Total breast meat was calculated as the sum of the P. major and P. minor weights, and the yield of each part was determined by division of the part weight by the individual live weight taken in the morning on the day of processing.

Plasma Nitric Oxide, Alpha-1-Acid Glycoprotein, and Carotenoid Analyses

Nitric oxide concentration (μM) in the plasma of blood samples was determined by colorimetric assay following the manufacturer's protocol (Cayman Chemical Company, Ann Arbor, MI). As final oxidation products of NO in vivo, the sum of both nitrate (NO_3^-) and nitrite (NO_2^-) were used to determine total NO. Prior to analysis, plasma samples were deproteinized using pre-rinsed Amicon® Ultra 30,000 MWCO filters (MilliporeSigma, Burlington, MA) to remove interfering proteins with a molecular weight greater than 30 kilodaltons, and samples were diluted 1:2 with assay buffer prior to beginning the assay. Alpha-1-acid glycoprotein concentration (mg/mL) was determined using a chicken AGP sandwich ELISA according to the manufacturer’s protocol (Life Diagnostics, West Chester, PA), and samples were diluted 1:10,000 with assay diluent prior to conducting the assay. Plasma carotenoids were determined

under yellow light using a method previously described by Allen et al. (1996). Briefly, plasma was diluted 1:10 in absolute ethanol, centrifuged to sediment protein, and transferred to a microtiter plate for measurement of absorbance by spectrophotometer (Molecular Devices, San Jose, CA). Absorbance was measured at 474 nm with background correction performed by subtracting absorbance at 530 nm.

Analysis of Plasma AA by High-Performance Liquid Chromatography

For determination of plasma AA, 100 µL plasma was deproteinated with an equal volume of 10% w/v trichloroacetic acid, incubated for 10 min on ice, and centrifuged at 12,000 × *g* for 20 min at 4°C. Then, 10 µL of the supernatant was derivatized using 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate reagent following the methods of Outlaw et al. (2023). The AA in the supernatant were separated using an Acquity UPLC H-Class Plus chromatography instrument (Waters Corporation, Milford, MA) equipped with a Waters Acquity UPLC BEH C18 column (1.7 µm, 2.1 mm × 150 mm; Waters Corporation, Milford, MA) and fluorescence detector to determine concentrations of individual AA.

Ileal Secretory IgA Analyses

Secretory IgA concentration (µg per g digesta) in ileal digesta was determined using a chicken IgA double antibody sandwich ELISA kit (Abnova, Taipei City, Taiwan) following the method previously described by Parsons et al. (2023). Briefly, sIgA was dissolved from lyophilized digesta into physiological saline, then filtered and stored at -20°C. Prior to analysis, samples were thawed and diluted 1:50 with assay diluent and the ELISA was conducted according to the manufacturer's protocol.

Statistical Analyses

Data were analyzed by one-way ANOVA for a randomized complete block design with pen as the experimental unit and pen location as a random blocking factor, with eight replicate pens of each treatment. Pre-planned contrasts were used to determine the main effects of challenge (– or +), dArg:dLys ratio (1.05 or 1.29), and their interaction in the 2 × 2 factorial arrangement of treatments, while polynomial contrasts were used to determine any linear or quadratic trends in increasing dArg:dLys concentration within the challenged group (0.81 to 1.53 dArg:dLys) for each dependent variable. Variables with significant quadratic responses were subsequently subjected to regression analyses to properly evaluate dose responses to dietary Arg and predict vertex values. In all cases, statistical significance was determined at $P \leq 0.05$, and data were analyzed using JMP Pro 17.2 software (SAS Institute, Cary, NC).

RESULTS

Diet Analyses

Calculated and analyzed values for the experimental grower diets as well as the common starter and finisher diets are shown in Table 5.1. As anticipated, analyzed total and free Arg concentration in the experimental grower diets increased proportionally as calculated digestible Arg values increased. The analyzed free Arg concentration in the 0.81 dArg:dLys diet was 0.03%, and concentration increased by 0.11% on average in each subsequent diet up to 0.70% for the 1.53 dArg:dLys diet.

15 to 22 d Performance (Acute Challenge)

There were no interactions between challenge status and dArg:dLys ratio during the acute challenge period ($P > 0.05$; Table 5.2). Challenge significantly impaired BWG by 23%, FI by

12%, and FCR by 21 points (15%), but there were no effects of Arg ($P > 0.05$) on any performance measurement when comparing 1.05 versus 1.29 dArg:dLys within the factorial treatment arrangement. However, within the challenged titration only, dArg:dLys quadratically improved BWG ($P = 0.004$), FI ($P = 0.026$), and FCR ($P = 0.001$).

22 to 29 d Performance (Challenge Recovery)

No interactions were observed between challenge status and dArg:dLys ratio during the challenge recovery period ($P > 0.05$; Table 5.3). Challenge significantly reduced BWG (2.4%) and FI (3.5%), but not FCR. However, 1.29 dArg:dLys improved FCR regardless of challenge status compared to 1.05 dArg:dLys ($P = 0.004$). Increasing dietary Arg for challenged birds quadratically increased BWG ($P = 0.002$) and FI ($P = 0.013$), and linearly decreased FCR ($P = 0.0003$).

11 to 39 d Performance (Cumulative)

There were no interactions between challenge status and dArg:dLys ratio for the cumulative performance period ($P > 0.05$; Table 5.4), which included the 10 d common finisher phase. Cumulative BWG remained reduced by challenge ($P = 0.033$), but FI and FCR were similar regardless of challenge status ($P > 0.05$). There were no effects of dArg:dLys ratio on overall performance measurements within the factorial treatment arrangement. However, within the challenged dArg:dLys titration, cumulative BWG, FI, and FCR were quadratically responsive ($P < 0.05$) to dArg:dLys ratio as Arg increased.

Regression Analyses

All performance parameters from the experimental phase that responded quadratically were subjected to regression analysis to estimate the dietary dArg:dLys ratio at each respective vertex (Table 5.5). Vertex values ranged from 1.09 dArg:dLys for FI during the challenge

recovery period (22 to 29 d) to 1.27 dArg:dLys to optimize FCR during the cumulative experimental grower period (11 to 29 d).

Plasma Nitric Oxide, Alpha-1-Acid Glycoprotein, and Carotenoid Concentrations

There were no interactions between challenge status and dArg:dLys ratio for any of the plasma measurements at 22 d ($P > 0.05$; Figure 5.1). As expected, plasma NO was increased ($P < 0.0001$) by challenge, increasing from 8.120 μM in unchallenged birds to 19.933 μM on average in challenged birds. For the dArg:dLys titration within the challenge group, there were no linear or quadratic trends with increasing Arg ($P > 0.05$). Plasma AGP concentration increased ($P = 0.027$) from 0.151 in unchallenged birds to 0.253 mg/mL in challenged birds, but there were no effects of dArg:dLys ratio within the challenge titration group. As anticipated, the enteric challenge model significantly decreased plasma carotenoid concentration, reducing concentration 4-fold from 10.638 μg/mL in unchallenged birds to 2.669 μg/mL in challenged birds.

Plasma Amino Acid Concentrations

At 22 d, there was one significant interaction for plasma serine (**Ser**) where 1.29 dArg:dLys decreased Ser concentration in the unchallenged birds but increased concentration in challenged birds. In the 2 × 2 factorial, enteric challenge significantly reduced plasma concentration of Arg ($P = 0.013$), asparagine (**Asn**; $P = 0.001$), glutamine (**Gln**; $P = 0.0002$), and tyrosine (**Tyr**; $P = 0.021$) (Tables 5.6 and 5.7). As expected, the higher 1.29 dArg:dLys ratio significantly increased plasma Arg concentration compared to 1.05 dArg:dLys ($P < 0.0001$). Plasma concentration of Orn was also increased by the higher Arg ratio ($P < 0.0001$), while Gln ($P = 0.001$) and Tyr ($P = 0.023$) concentrations were decreased. Within the challenged titration group, increasing Arg quadratically increased plasma concentrations of Arg ($P = 0.006$) and Orn

($P = 0.003$), but linearly increased plasma Cit ($P = 0.002$). Plasma Asn ($P = 0.009$), Gln ($P < 0.0001$), Thr ($P = 0.001$), Ala ($P = 0.0002$), and Tyr ($P = 0.002$) concentrations linearly decreased, while lysine (**Lys**) quadratically decreased ($P = 0.001$).

Ileal Secretory IgA Concentration

No interactions were observed between challenge status and dArg:dLys ratio on the concentration of sIgA determined in the ileal digesta at 22 d ($P > 0.05$; Figure 5.2). Secretory IgA concentration was significantly reduced by challenge ($P = 0.001$), but there were no significant trends observed with increasing Arg within the challenge group fed the dArg:dLys titration ($P > 0.05$).

Processing

No challenge status by dArg:dLys ratio interactions were observed for any processing measurement ($P > 0.05$; Table 5.8). Chilled carcass yield was significantly increased by 1.29 dArg:dLys compared to 1.05 dArg:dLys ($P = 0.031$), but no additional effects of dArg:dLys ratio were observed. Tender weight as well as total breast meat weight were decreased in challenged birds ($P < 0.05$), whereas drum yield increased with challenge by approximately 0.10% ($P = 0.046$).

DISCUSSION

This experiment was conducted to assess whether dietary Arg needs of broilers are influenced during enteric challenge (unchallenged or challenged × 1.05 or 1.29 dArg:dLys) and to establish optimal responses of challenged broilers to dietary Arg based on a titration of 0.81 to 1.53 dArg:dLys. Regarding growth performance, BWG and FI of challenged birds were reduced by 9.5 and 6.1%, respectively, during the experimental grower phase (acute challenge +

challenge recovery period), and FCR was increased by 5.5 points (3.8%). An economic analysis by Skinner et al. (2010) estimated that subclinical NE infections cause a 12.0% reduction in BW and a 10.9% increase in FCR compared with healthy birds; however, it should be considered that similar reductions in growth performance may also be achieved in models using *Eimeria* spp. alone, necessitating the confirmation of subclinical NE with gross lesions or by other means (Kaldhusdal and Hofshagen, 1992; Castro et al., 2020). Interestingly, our study did not result in any gross lesions in the jejunum compared to the unchallenged, sham-inoculated birds at 22 d (data not shown), which could have been related to the specific *Eimeria* spp. and *C. perfringens* isolates used, the timing of the scoring relative to disease progression, or other factors. Regardless, there was a lack of a key pathological finding for the diagnosis of subclinical NE in this trial, despite *C. perfringens* administration (Kaldhusdal and Hofshagen, 1992).

Several indicators of intestinal function and inflammation responded to the enteric challenge employed in this study. Plasma carotenoids, for example, are indicators of intestinal disruption proportionally depressed in response to epithelial damage and can be used as sensitive indirect evaluators of enteric challenge (Conway et al., 1993; Hernández-Velasco et al., 2014; Rochell et al., 2016a,b). In our study, plasma carotenoid concentration decreased 4-fold in challenged birds, and it is interesting to note that baseline unchallenged control levels were higher than other published results (Allen and Fetterer, 2000; Rochell et al., 2016a,b; Rochell et al., 2017). This was likely due to the inclusion of corn gluten meal in the basal diet to achieve the lowest target concentration of Arg.

Plasma NO and AGP concentration were also responsive to enteric challenge. Notably, NO concentration doubled on average in challenged birds compared to unchallenged controls, likely due to its role as a complex regulator of the inflammatory response associated with

parasitic infection and the pathogenesis of intestinal disorder (Allen, 1997; Park et al., 2008; Pirali Kheirabadi et al., 2011). Plasma concentration of AGP, a positive acute-phase protein indicative of systemic inflammation that has been linked to gut barrier failure associated with both *Eimeria* infection and NE (Adler et al., 2001; Chen et al., 2015; Dao et al., 2022), also increased with challenge. This may have been related to the increase in NO, which along with pro-inflammatory cytokines and glucocorticoids, can influence the acute phase protein response (Gruys et al., 2005).

The enteric challenge employed in the current study also induced changes in average ileal sIgA secretion. Secretory IgA is a protective immunoglobulin on the surface of the intestinal mucosa that helps to neutralize toxins, regulate the symbiotic relationship between commensal bacteria and the host, and particular to our study, helps prevent opportunistic bacterial and parasitic pathogens from adhering to or penetrating the epithelium (Davis et al., 1978; Corthésy, 2013). Results on whether ileal sIgA concentrations increase or decrease in response to enteric challenge models in broilers have been mixed, but it has been suggested that this could be due in part to varying degrees of damage caused to intestinal tissue with different disease models and sampling protocols (Parker et al., 2007; Zhang et al., 2017; Parsons, 2023; Souza et al., 2024). Indeed, SIgA production may depend on the overall microbial load, including existing microbiota and that introduced in the challenge model, along with the intestinal segment and time point from which concentration was measured (Zhang et al., 2017).

Several studies have also shown that the circulating free AA pool of chickens can be responsive to enteric challenge (Rochell et al., 2016b; Izadi Yazdanabadi et al., 2020; Lee et al., 2024). In broilers, Rochell et al. (2016b) showed that AA concentrations were linearly or quadratically affected by increasing inoculation dose of *E. acervulina*; specifically, plasma

concentrations of Arg and Tyr decreased linearly and Gln and Asn decreased quadratically, which is in agreement with the results of our study. Specifically for plasma Arg, it has been hypothesized that the decrease in concentration of this AA due to enteric challenge may be from an increased metabolic demand for Arg as a precursor for NO; however, at least with *E. acervulina* infection, a study by Allen and Fetterer (2000) suggests that this is more likely due to the anorexia and malabsorption inherent with enteric disease.

Within challenged birds, increasing the dietary dArg:dLys ratio from 0.81 to 1.53 had significant effects on broiler growth performance, processing characteristics, and plasma AA concentrations evaluated in this trial. In the acute challenge period (15 to 22 d; 0 to 7 d post-infection), quadratic responses in BWG, FI, and FCR among birds fed the Arg titration show that these performance measurements are responsive to dietary Arg during acute enteric infection. Specifically, the greatest improvement in BWG and FCR due to Arg in this phase was 33 g and 10 points, respectively, with the optimum values predicted to be achieved using a dArg:dLys ratio of 1.18 and 1.20. Other studies evaluating Arg concentration during this similar period of challenge also show that Arg can influence broiler performance responses; however, most have included a limited range of Arg ratios that make it difficult to assess polynomial trends across levels (Laika and Jahanian, 2017). Castro et al. (2020) used a similar trial design to our study and observed linear increases in BWG using 5 Arg ratios (0.88, 0.97, 1.05, 1.14, and 1.22 dArg:dLys) in Cobb broilers 0 to 6 d after an *Eimeria* spp. gavage. However, they found no differences in FI or FCR. Alternatively, Tan et al. (2014) reported that dietary Arg linearly improved FCR in broilers from 0 to 7 d after coccidiosis challenge, but broilers were fed only 3 Arg ratios and the linear response was largely influenced by the lowest (deficient) level.

Differences in findings among studies may be due to challenge model methodology, Arg concentrations chosen, and genetic strain.

The lack of significant main effects or interactions of Arg on growth performance in the factorial analysis of our study may have been due to the Arg ratios selected for the arrangement. For example, in the case of BWG the dArg:dLys ratio of 1.17 resulted in higher numeric values in each phase compared to 1.29. Thus, perhaps 1.05 and 1.17 dArg:dLys × unchallenged or challenged could have been better suited for the factorial analysis. Even so, Fathima et al. (2024) did not find any significant benefit of 25% Arg supplementation above the breeder recommendation within 7 d of either *Eimeria*/*C. perfringens* or sham gavages except for a reduction in FI.

The quadratic effect of dietary Arg on BWG and FI observed during the acute challenge phase persisted during the recovery period (22 to 29 d; 7 to 14 d post-infection), with the highest BWG predicted to be achieved at a dArg:dLys ratio of 1.20. Interestingly, the FCR response for this phase was linear. Although the effect size of improvement in FCR among challenged birds (approximately 5 points) was smaller than that observed in the acute phase, the linear response to diet indicates that the dArg:dLys ratios in the titration were not high enough to achieve a breakpoint for the estimation of a requirement in this study. In the aforementioned study by Castro et al. (2020) the authors reported a linear increase in FI during the recovery phase (7 to 14 d post *Eimeria* spp. gavage) with increasing Arg and a tendency for quadratic improvement in BWG ($P = 0.060$), but no change in FCR. However, unchallenged birds fed the same 5 Arg concentrations did show a linear improvement in FCR. Thus, perhaps differences in results compared to our study can be attributed to differences in the type and severity of the challenge models employed. Positive effects of Arg on growth performance could be due to a variety of

reasons including its influence on anabolic growth factors, energy and protein metabolism, intestinal health, and immune function.

The lack of plasma NO response due to dArg:dLys ratio in this study is notable. This is because L-Arg is the only known metabolic precursor for NO, synthesized with Cit by inducible NOS enzymes during periods of innate immune activation by inflammatory stimuli (Fernandes and Murakami, 2010). Thus, particularly during *Eimeria* and NE infections, a key metabolic demand of Arg is for production of NO (Rochell et al., 2017). There is strong evidence to suggest that dietary Arg can significantly influence plasma NO concentrations in broilers under certain conditions (Takahashi et al., 1999; Kwak et al., 2001; Khajali et al., 2011; Basoo et al., 2012); however, the results of studies specifically evaluating enteric disease models have been less consistent (Allen, 1999; Rochell et al., 2017; Castro et al., 2020; Izadi Yazdanabadi et al., 2020). Lack of reported NO responses to dietary Arg during intestinal challenge could be due to sampling time, as studies have shown that detectable increases in plasma NO from *Eimeria* infection occur after a lag time that is consistent with an inducible system of immune response (Allen, 1997; Pirali Kheirabadi et al., 2011). Furthermore, Takahashi et al. (1999) showed that the detectable NO response to systemic inflammation induced by intraperitoneal lipopolysaccharide (**LPS**) injection can both peak and return to baseline levels mostly within 24 h after injection. Furthermore, Chapman and Wideman (2006) reported that colorimetric assays for determination of total plasma NO concentrations similar to that used in the current study adequately reflect whole-body synthesis, but may not be sensitive enough to detect small yet biologically relevant quantities. Perhaps, as the NO demand for birds experiencing enteric challenge is lower and less acute that induced by LPS injection (Allen, 1997; Chapman and Wideman, 2006; Rochell et al., 2017), broilers in our study had a better opportunity to mobilize

other sources of Arg (i.e., body proteins) to synthesize NO if dietary Arg availability was limited.

The effect of dietary Arg on plasma AGP concentration in chickens has not been extensively studied. A previous study in our lab showed that plasma AGP was quadratically responsive to increasing dArg:dLys within 24 h of an intradermal (i.d.) LPS injection (Anderson, Chapter 3). Similarly, Takahashi et al. (1999) showed that chicks fed an Arg-sufficient or -excess diet had a higher ratio of AGP production within 24 h of LPS injection compared to those fed an Arg-deficient diet. In that study, AGP concentration also coincided with NO production at 6 and 10 h post-injection. However, the lack of significant AGP response to dietary dArg:dLys in our current study agrees with that of Dao et al. (2022) using a subclinical NE model, and therefore may be attributed to the differences in inflammatory stimuli used from the previous studies or the time point measured, as AGP levels in Takahashi et al. (1999) had already normalized by 24 h.

Secretory IgA responses to dietary Arg in chickens reported in the literature have been inconsistent. In unchallenged birds, Ruan et al. (2020) and Yu et al. (2018) showed that increasing Arg can increase ileal content of sIgA in both meat- and layer-type chickens. However, in broilers challenged with *Eimeria* and/or *C. perfringens*, Zhang et al. (2017) showed that Arg supplementation enhanced jejunal but not ileal sIgA, while Tan et al. (2014) reported that increasing Arg concentration inconsistently influenced jejunal mucosal sIgA content. It has been suggested that NO and IL-10 production regulate sIgA secretion, potentially linking sIgA secretion with dietary Arg concentration (Viana et al., 2013; Zhang et al., 2017). Furthermore, Ruan et al. (2020) showed that dietary Arg increased the relative abundance of the *Candidatus Arthromitus* genus of bacteria in ileum, which can modulate the induction of IgA plasma cells and intestinal sIgA secretion (Umesaki et al., 1999; Robino et al., 2019). However, the results of

our study do not support these data, as dArg:dLys was not shown to influence ileal IgA secretion in our trial. Possibly, the enteric challenge disrupted the intestinal microbiota and/or the epithelium enough to diminish the potential for Arg to modulate the sIgA response, as host-microbial interactions are often considered the predominant factor driving production of sIgA (Parsons, 2023). Alternatively, the time point and/or intestinal segment measured may have been insufficient to capture the effect of Arg on sIgA.

The plasma free AA pool, although very small compared to other AA pools, can be a useful tool to provide an overall view of AA behavior as it reflects the rate of AA appearance and disappearance from the plasma (Cynober, 2002). In our study, differences in plasma concentration of several AA indicate that increasing dArg:dLys ratio can clearly alter AA metabolism in broilers. Increases in plasma Arg concomitant with dietary Arg concentration confirmed expected increases of bioavailable Arg for metabolic use. Plasma concentrations of other Arg-related AA were also affected by dietary dArg:dLys ratio. For example, Orn, a product of Arg catabolism by arginase, increased in the plasma quadratically as dietary Arg increased in challenged birds but also regardless of challenge status (1.05 versus 1.29 dArg:dLys). Ornithine is a substrate for the synthesis of polyamines, but it is unknown whether higher Orn levels in our study were synthesized to repair intestinal mucosal damage or as a response of the birds to catabolize and eliminate excess Arg (Morris, 2007; Wang et al., 2015; Tan et al., 2024). Ornithine is also a metabolic precursor to Pro, required for collagen synthesis, and thus it can promote wound healing of connective tissues (Wu and Morris, 1998; Li and Wu, 2018; Aydin et al., 2019). Plasma concentrations of Pro, however, did not change in our study.

Citrulline is another product of Arg catabolism, synthesized by nitric oxide synthase enzymes which can compete with arginase for Arg as a substrate (Chang et al., 1998; Wu and

Morris, 1998). The linear increase in plasma Cit with increased dArg:dLys ratio in challenged birds is intriguing, as this suggests that NO as a product of this reaction would also be increased. As mentioned above, dietary Arg concentration had no influence on plasma NO in this trial, potentially indicating that Cit may be a more persistent, indirect indicator of NO synthesis. However, this idea would require further investigation as Cit concentration can also be altered by other metabolic pathways (Tsikas et al., 2004; Pérez-Neri et al., 2020).

Additional plasma AA affected by dArg:dLys in our study include Gln, Thr, Asn, Ala, Tyr, and Ser. Glutamine is an important energy source for enterocytes, a precursor for glutathione, and can also serve as a major precursor for the enteral synthesis of Arg (Wu and Morris, 1998; Sifa et al., 2008; Bortoluzzi et al., 2018). Plasma concentrations of Gln were linearly decreased with increased dArg:dLys in challenged birds in our study and as a main effect of dArg:dLys ratio in the analysis of the factorial. Possibly, this could correspond to results of Brugaletta et al. (2023), who reported reductions in concentration of hepatic glutathione with Arg supplementation. Notably, Gln has also been found to be a physiological regulator of NO production in humans and can reduce the intracellular concentrations of Arg (Marques et al., 2013; Pérez-Neri et al., 2020). Nitric oxide can also reciprocally regulate Gln synthesis through Tyr nitration of glutamine synthetase (Muscoli et al., 2013; Pérez-Neri et al., 2020). In general, changes in the analyzed concentrations of plasma AA in our study from dietary dArg:dLys ratio could be due to physiological shifts in metabolism that result from either deficiency or excess of Arg. For example, Ala can be a vehicle for inter-organ transport of the carbon and nitrogen atoms from AAs (Wu et al., 1989). Another possibility is that the observed changes in AAs reflect the influence that Arg has on the immune system. In pathological disease situations, defense and recovery efforts can modify the requirements for certain AAs; thus, the

metabolism of Thr, for instance, may change as it is a major component of mucin and immunoglobulins (Faure et al., 2007; Bortoluzzi et al., 2018).

Due to logistical constraints, only birds from the 2 × 2 factorial arrangement of treatments were processed at 39 d. Interestingly, 1.29 dArg:dLys increased chilled carcass yield compared to birds fed 1.05 regardless of challenge status, and the yield difference between main effect means was 0.30%. This difference resulted from several numeric increases in individual parts yields, with the greatest numerical increase occurring for thigh yield (0.23%; $P = 0.066$). Sirathonpong et al. (2019) fed broilers increasing Arg:Lys ratios ranging from 0.85 to 1.26% from 1 to 35 d and reported that carcass and breast meat yield linearly improved as Arg ratio increased. However, other studies have reported no differences in carcass or parts yields between broilers fed the breeder recommended dArg:dLys ratio compared to those supplemented up to 30% higher (Zampinga et al., 2019; Brugaletta et al., 2023). Nonetheless, the 1.05 dArg:dLys ratio in our study appeared to be below the Arg requirement to maximize carcass yield.

In conclusion, broilers subjected to enteric challenge with *Eimeria* spp. and *C. perfringens* experienced impaired growth performance that was accompanied by other physiological changes including higher concentration of plasma NO and AGP. The dArg:dLys ratios that optimized growth performance of challenged birds in our study ranged from 1.09 to 1.27, indicating that growth performance of challenged broilers, particularly FCR, could be improved by feeding Arg ratios that are higher than current recommendations. However, it remains unclear whether these responses to Arg were influenced by challenge status due to the lack of interaction between challenge and limited Arg ratios evaluated in the factorial (1.05 versus 1.29). Overall, these results indicate that dietary Arg may be used as part of an effective strategy to ameliorate some of the negative effects of enteric challenges in broilers.

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Table 5.1. Ingredient and calculated nutrient composition of diets (% as-fed, unless otherwise noted).

Item	Common Starter (0 to 11 d)	Experimental Grower Diet A (11 to 29 d)[1]	Common Finisher (29 to 39 d)
Corn	56.495	61.280	67.718
Soybean meal	34.403	13.035	28.616
DDGS[2]	3.000	13.741	-
Corn gluten meal	1.500	7.000	-
Fat, animal	0.925	0.705	1.181
Calcium carbonate	1.143	0.950	0.734
Dicalcium phosphate	1.008	0.629	0.482
Salt	0.326	0.216	0.363
Potassium carbonate	-	0.140	-
Sodium sesquicarbonate	0.059	0.064	0.045
Inert filler (sand)	-	0.800	-
L-Lysine·HCl	0.264	0.531	0.168
L-Methionine	0.389	0.299	0.343
Threonine fermentate	0.168	0.199	0.119
L-Isoleucine	-	0.097	-
L-Arginine	0.098	-	0.043
L-Valine	0.009	0.070	0.005
L-Tryptophan	-	0.033	-
Trace mineral premix[3]	0.100	0.100	0.100
Vitamin premix[4]	0.100	0.100	0.050
Choline chloride	-	-	0.020
Xylanase[5]	0.008	0.008	0.008
Phytase[6]	0.005	0.005	0.005
Calculated nutrients			
ME, kcal/kg	2,976	3,053	3,100
CP	23.70	20.53	19.76
Digestible Lys	1.32	1.10	1.08
Digestible Arg	1.41	0.89	1.17
Analyzed nutrients[7,8]			
CP	24.71	21.30	20.32
Total Lys	1.54	1.25	1.26
Total Arg	1.63	1.09	1.35

[1]Cystalline L-Arginine was added to Diet A (calculated dArg:dLys ratio of 0.81) at the expense of inert filler in order to meet calculated dArg:dLys ratios for Diets B-G (0.93, 1.05, 1.17, 1.29, 1.41, and 1.53, respectively).
[2]Distillers dried grains with solubles.
[3]Mineral premix includes per kg of diet: Mn (manganese sulfate), 120 mg; Zn (zinc sulfate, 100 mg; Fe (ferrous sulfate), 30 mg; Cu (basic copper chloride), 8 mg; I (stabilized ethylenediamine dihydroiodide), 1.4 mg; Se (sodium selenite), 0.3 mg.
[4]Vitamin premix includes per kg of diet: Vitamin A, 18,739 IU; Vitamin D3, 6,614 IU; Vitamin E, 66 IU; niacin, 88 mg; d-pantothenic acid (d-calcium pantothenate), 31 mg; riboflavin, 22 mg; Vitamin B6 (pyridoxine hydrochloride), 7.7 mg; thiamine (thiamine mononitrate), 5.5 mg;

menadione (menadione sodium bisulfite complex), 4 mg; folic acid, 2.6 mg; biotin, 0.18 mg; Vitamin B12, 0.03 mg.
[5]Econase XT (AB Vista Feed Ingredients, Marlborough, UK).
[6]Quantum Blue 10G (AB Vista Feed Ingredients, Marlborough, UK).
[7]The analyzed total Arg:Lys ratios for experimental grower diets B-G were 0.96, 1.05, 1.14, 1.24, 1.34, and 1.41, respectively.
[8]The analyzed free Arg concentration for experimental grower diets A-G was 0.03, 0.13, 0.25, 0.35, 0.47, 0.56, and 0.70%, respectively.

Table 5.2. Growth performance of Ross 708 male broilers from 15 to 22 d (acute challenge period) fed increasing concentrations of Arg from 11 to 39 d post-hatch[1,2].

Item	15 d BW, kg	22 d BW, kg	BWG, kg	FI, kg	FCR, kg:kg
Unchallenged –1.05	0.569	1.122	0.554	0.806	1.457
Unchallenged – 1.29	0.574	1.134	0.560	0.809	1.443
Challenged – 0.81	0.562	0.963	0.401	0.697	1.745
Challenged – 0.93	0.567	0.998	0.421	0.714	1.665
Challenged – 1.05	0.570	1.003	0.432	0.718	1.662
Challenged – 1.17	0.573	1.007	0.434	0.713	1.647
Challenged – 1.29	0.573	1.001	0.428	0.708	1.659
Challenged – 1.41	0.578	0.997	0.418	0.699	1.675
Challenged – 1.53	0.572	0.995	0.423	0.699	1.666
SEM	0.003	0.008	0.007	0.007	0.016
Factorial main effect of Challenge					
Unchallenged	0.571	1.128[a]	0.557[a]	0.807[a]	1.450[b]
Challenged	0.571	1.002[b]	0.430[b]	0.713[b]	1.661[a]
SEM	0.003	0.008	0.007	0.007	0.016
Factorial main effect of Arg ratio					
1.05	0.569	1.062	0.493	0.762	1.560
1.29	0.573	1.067	0.494	0.758	1.551
SEM	0.003	0.008	0.007	0.007	0.016
Factorial *P*-values					
Main effect of Challenge	0.936	***<0.0001***	***<0.0001***	***<0.0001***	***<0.0001***
Main effect of Arg ratio	0.213	0.542	0.849	0.636	0.585
Challenge × Arg ratio	0.628	0.413	0.457	0.378	0.719
Arg Titration *P*-values					
Linear	***0.001***	***0.043***	0.152	0.367	***0.012***
Quadratic	0.069	***0.002***	***0.004***	***0.026***	***0.001***

[a-b]Values in columns not sharing the same letter are significantly different ($P \leq 0.05$).

[1]Values are least square means of 8 replicate pens per treatment with 25 birds per pen.

[2]Birds were challenged by oral gavage with a 10-fold dose of a live coccidiosis vaccine at 15 d as well as a 10^7 and 10^6 CFU dose of *C. perfringens* at 19 and 20 d, respectively.

Table 5.3. Growth performance of Ross 708 male broilers from 22 to 29 d (challenge recovery period) fed increasing concentrations of Arg from 11 to 39 d post-hatch[1,2].

Item	22 d BW, kg	29 d BW, kg	BWG, kg	FI, kg	FCR, kg:kg
Unchallenged –1.05	1.122	1.849	0.727	1.102	1.510
Unchallenged – 1.29	1.134	1.877	0.743	1.102	1.471
Challenged – 0.81	0.963	1.660	0.697	1.068	1.498
Challenged – 0.93	0.998	1.708	0.710	1.065	1.479
Challenged – 1.05	1.003	1.721	0.719	1.074	1.485
Challenged – 1.17	1.007	1.732	0.726	1.072	1.475
Challenged – 1.29	1.001	1.716	0.715	1.052	1.462
Challenged – 1.41	0.997	1.714	0.718	1.056	1.451
Challenged – 1.53	0.995	1.697	0.702	1.029	1.454
SEM	0.008	0.012	0.007	0.008	0.010
Factorial main effect of Challenge					
Unchallenged	1.128[a]	1.863[a]	0.735[a]	1.102[a]	1.490
Challenged	1.002[b]	1.718[b]	0.717[b]	1.063[b]	1.474
SEM	0.008	0.012	0.007	0.008	0.010
Factorial main effect of Arg ratio					
1.05	1.062	1.785	0.723	1.088	1.497[a]
1.29	1.067	1.796	0.729	1.077	1.466[b]
SEM	0.008	0.012	0.007	0.008	0.010
Factorial *P*-values					
Main effect of Challenge	***<0.0001***	***<0.0001***	***0.015***	***<0.0001***	0.108
Main effect of Arg ratio	0.542	0.328	0.372	0.160	***0.004***
Challenge × Arg ratio	0.413	0.158	0.174	0.151	0.442
Arg Titration *P*-values					
Linear	***0.043***	***0.057***	0.474	***0.0003***	***0.0003***
Quadratic	***0.002***	***0.0001***	***0.002***	***0.013***	0.872

[a-b]Values in columns not sharing the same letter are significantly different ($P \leq 0.05$).

[1]Values are least square means of 8 replicate pens per treatment with 25 birds per pen.

[2]Birds were challenged by oral gavage with a 10-fold dose of a live coccidiosis vaccine at 15 d as well as a 10^7 and 10^6 CFU dose of *C. perfringens* at 19 and 20 d, respectively.

Table 5.4. Growth performance of Ross 708 male broilers from 11 to 39 d fed increasing concentrations of Arg from 11 to 39 d post-hatch[1,2].

Item	11 d BW, kg	39 d BW, kg	BWG, kg	FI, kg	FCR, kg:kg
Unchallenged –1.05	0.340	3.093	2.753	4.106	1.593
Unchallenged – 1.29	0.343	3.096	2.753	4.112	1.585
Challenged – 0.81	0.343	2.983	2.641	4.039	1.621
Challenged – 0.93	0.343	3.033	2.690	4.041	1.618
Challenged – 1.05	0.342	3.064	2.722	4.094	1.580
Challenged – 1.17	0.343	3.094	2.751	4.080	1.578
Challenged – 1.29	0.342	3.042	2.700	4.044	1.578
Challenged – 1.41	0.344	3.052	2.708	4.067	1.584
Challenged – 1.53	0.341	3.006	2.664	3.976	1.589
SEM	0.002	0.020	0.019	0.027	0.008
Factorial main effect of Challenge					
Unchallenged	0.341	3.094[a]	2.753[a]	4.108	1.589
Challenged	0.342	3.053[b]	2.711[b]	4.069	1.579
SEM	0.002	0.020	0.019	0.027	0.008
Factorial main effect of Arg ratio					
1.05	0.341	3.078	2.738	4.100	1.587
1.29	0.342	3.069	2.727	4.078	1.582
SEM	0.002	0.020	0.019	0.027	0.008
Factorial *P*-values					
Main effect of Challenge	0.685	***0.041***	***0.033***	0.155	0.224
Main effect of Arg ratio	0.374	0.636	0.578	0.411	0.536
Challenge × Arg ratio	0.226	0.520	0.573	0.316	0.706
Arg Titration *P*-values					
Linear	0.736	0.434	0.405	0.200	***0.0004***
Quadratic	0.972	***0.0001***	***0.0001***	***0.010***	***0.001***

[a-b]Values in columns not sharing the same letter are significantly different ($P \leq 0.05$).

[1]Values are least square means of 8 replicate pens per treatment with 25 birds per pen.

[2]Birds were challenged by oral gavage with a 10-fold dose of a live coccidiosis vaccine at 15 d as well as a 10^7 and 10^6 CFU dose of *C. perfringens* at 19 and 20 d, respectively.

Table 5.5. Parameters of linear and quadratic regression of Ross 708 male broilers fed increasing concentrations of Arg from 11 to 39 d of age[1,2].

Item	Equation	*P*-value	R^2	SE Intercept	SE Slope
Linear					
15 to 22 d – Acute Challenge Period					
15 d BW, kg	$y = 0.55078 + 0.00017x$	***0.0008***	0.149	0.006	0.00005
22 to 29 d – Challenge Recovery Period					
FCR, kg:kg	$y = 1.55633 - 0.00069x$	***<0.0001***	0.208	0.019	0.00016
Quadratic					
15 to 22 d – Acute Challenge Period				Vertex	Arg at Vertex
22 d BW, kg	$y = 0.17686 + 0.01470x - 0.00006x^2$	***<0.0001***	0.236	1.054	119
BWG, kg	$y = -0.32266 + 0.01363x - 0.00006x^2$	***0.0003***	0.210	0.481	118
FI, kg	$y = 0.16210 + 0.01014x - 0.00004x^2$	***0.0004***	0.200	0.750	116
FCR, kg:kg	$y = 3.15095 - 0.02635x + 0.00011x^2$	***<0.0001***	0.260	1.571	120
22 to 29 d – Challenge Recovery Period					
BWG, kg	$y = 0.41048 + 0.00531x - 0.00002x^2$	***0.0002***	0.223	0.728	120
FI, kg	$y = 0.81489 + 0.00490x - 0.00002x^2$	***0.0006***	0.192	1.082	109
11 to 29 d – Cumulative Grower Period					
29 d BW, kg	$y = 0.58739 + 0.02001x - 0.00008x^2$	***<0.0001***	0.292	1.782	119
BWG, kg	$y = 0.24047 + 0.02009x - 0.00008x^2$	***<0.0001***	0.297	1.440	119
FI, kg	$y = 1.18561 + 0.01608x - 0.00007x^2$	***0.0002***	0.224	2.102	114
FCR, kg:kg	$y = 2.10158 - 0.00993x + 0.00004x^2$	***<0.0001***	0.519	1.471	127

[1]Values are least square means of 8 replicate pens per treatment with 25 birds per pen.

[2]Birds were challenged by oral gavage with a 10-fold dose of a live coccidiosis vaccine at 15 d as well as a 10^7 and 10^6 CFU dose of *C. perfringens* at 19 and 20 d, respectively.

Table 5.6. Plasma concentrations of Arg-related and essential amino acids (μmol/L) at 22 d of Ross 708 male broilers fed increasing concentrations of Arg from 11 to 39 d post-hatch[1,2].

	Arg	Orn	Cit	Pro	Lys	Met	Thr	Val	Ile	Leu	His	Phe
Unchallenged – 1.05	220.8	22.2	6.7	351.5	96.8	64.7	524.2	98.3	54.3	175.3	32.8	92.6
Unchallenged – 1.29	343.4	59.8	6.4	328.8	114.7	72.1	516.4	94.7	53.1	167.6	32.0	85.3
Challenged – 0.81	98.4	9.2	6.7	318.2	251.1	64.8	634.7	96.9	56.1	175.9	42.2	84.8
Challenged – 0.93	141.5	10.3	6.1	350.5	136.4	70.3	513.7	101.7	60.6	190.1	36.9	95.6
Challenged – 1.05	198.0	19.3	6.7	330.6	129.7	63.0	447.3	96.8	56.3	175.1	37.2	90.6
Challenged – 1.17	261.7	36.5	6.3	319.6	160.1	66.2	473.5	92.5	53.6	157.0	37.9	78.1
Challenged – 1.29	285.9	43.0	7.7	293.0	133.1	61.8	444.1	93.7	55.5	151.9	35.7	84.5
Challenged – 1.41	287.7	64.0	8.0	324.7	131.6	66.5	454.7	96.0	57.2	168.5	38.4	93.1
Challenged – 1.53	319.8	87.3	8.7	318.4	130.5	74.0	404.7	96.6	57.0	171.7	36.7	91.1
SEM	15.740	5.409	0.679	26.510	16.550	5.476	41.590	5.097	3.052	10.700	2.255	3.735
Factorial main effect of Challenge												
Unchallenged	282.1[a]	41.0	6.5	340.1	105.7	68.4	520.3	96.5	53.7	171.5	32.4	89.0
Challenged	242.0[b]	31.1	7.2	311.8	131.4	62.4	445.7	95.2	55.9	163.5	36.4	87.5
SEM	15.740	5.154	0.647	26.510	16.550	5.476	41.590	5.097	3.052	10.700	2.255	3.735
Factorial main effect of Arg ratio												
1.05	209.4[b]	20.8[b]	6.7	341.0	113.2	63.9	485.8	97.6	55.3	175.2	35.0	91.6
1.29	314.6[a]	51.4[a]	7.1	310.9	123.9	67.0	480.2	94.2	54.3	159.7	33.9	84.9
SEM	15.740	5.154	0.647	26.510	16.550	5.476	41.590	5.097	3.052	10.700	2.255	3.735
Factorial *P*-values												
Main effect of Chall.	***0.013***	0.060	0.293	0.289	0.109	0.272	0.078	0.799	0.476	0.458	0.081	0.702
Main effect of Arg	***<0.0001***	***<0.0001***	0.552	0.260	0.503	0.574	0.894	0.509	0.727	0.153	0.620	0.078
Challenge × Arg	0.275	0.185	0.328	0.778	0.647	0.435	0.956	0.966	0.943	0.474	0.884	0.876
Arg Titration *P*-values												
Linear	***<0.0001***	***<0.0001***	***0.002***	0.529	***<0.0001***	0.514	***0.001***	0.563	0.755	0.167	0.214	0.695
Quadratic	***0.006***	***0.003***	0.154	0.809	***0.001***	0.278	0.104	0.580	0.575	0.194	0.251	0.224

[a-b]Values in columns not sharing the same letter are significantly different ($P \leq 0.05$).

[1]Values are least square means of 8 replicate pens per treatment of 1 bird per pen.

[2]Birds were challenged by oral gavage with a 10-fold dose of a live coccidiosis vaccine at 15 d as well as a 10^7 and 10^6 CFU dose of *C. perfringens* at 19 and 20 d, respectively.

Table 5.7. Plasma concentrations of non-essential amino acids (μmol/L) at 22 d of Ross 708 male broilers fed increasing concentrations of Arg from 11 to 39 d post-hatch[1,2].

	Gln	Asn	Tau	Gly	Ser	Asp	Glu	Ala	Tyr
Unchallenged – 1.05	612.4	110.0	102.6	526.9	322.0	34.1	370.1	364.5	173.9
Unchallenged – 1.29	527.0	105.1	87.9	478.9	307.5	39.3	402.7	359.0	165.8
Challenged – 0.81	605.0	98.8	58.5	500.7	428.6	31.1	291.4	471.4	179.8
Challenged – 0.93	496.8	93.7	82.7	459.1	363.1	39.8	284.4	440.3	156.0
Challenged – 1.05	515.0	89.8	79.0	467.2	321.6	31.1	346.1	419.2	165.4
Challenged – 1.17	466.5	87.9	82.0	445.7	358.9	34.7	294.9	369.2	150.9
Challenged – 1.29	414.4	80.2	81.8	497.8	389.9	32.1	383.0	380.0	129.6
Challenged – 1.41	375.3	78.6	64.1	469.2	347.9	28.6	347.3	324.6	149.0
Challenged – 1.53	405.8	81.3	75.9	460.1	359.0	29.2	333.1	350.1	142.6
SEM	26.890	6.433	11.790	27.630	20.480	2.729	31.200	30.060	9.398
Factorial main effect of Challenge									
Unchallenged	569.7[a]	107.5[a]	95.2	502.9	314.7	36.7	386.4	361.8	169.8[a]
Challenged	464.7[b]	85.0[b]	80.4	482.5	355.8	31.6	364.6	399.6	147.5[b]
SEM	26.890	6.433	11.790	27.630	20.480	2.729	31.200	30.060	9.398
Factorial main effect of Arg ratio									
1.05	563.7[a]	99.9	90.8	497.0	321.8	32.6	358.1	391.9	169.7[a]
1.29	470.7[b]	92.6	84.9	488.4	348.7	35.7	392.8	369.5	147.7[b]
SEM	26.890	6.433	11.790	27.630	20.480	2.729	31.200	30.060	9.398
Factorial *P*-values									
Main effect of Chall.	***0.0002***	***0.001***	0.213	0.463	***0.050***	0.065	0.488	0.214	***0.021***
Main effect of Arg	***0.001***	0.261	0.618	0.755	0.195	0.255	0.270	0.460	***0.023***
Challenge × Arg	0.780	0.717	0.460	0.160	***0.048***	0.434	0.945	0.578	0.145
Arg Titration *P*-values									
Linear	***<0.0001***	***0.009***	0.775	0.628	0.120	0.065	0.086	***0.0002***	***0.002***
Quadratic	0.110	0.514	0.205	0.620	0.055	0.294	0.395	0.400	0.156

[a-b]Values in columns not sharing the same letter are significantly different ($P \leq 0.05$).

[1]Values are least square means of 8 replicate pens per treatment of 1 bird per pen.

[2]Birds were challenged by oral gavage with a 10-fold dose of a live coccidiosis vaccine at 15 d as well as a 10^7 and 10^6 CFU dose of *C. perfringens* at 19 and 20 d, respectively.

Table 5.8. Processing characteristics of Ross 708 male broilers fed increasing concentrations of Arg from 11 to 39 d post-hatch[1,2,3].

	Chilled Carcass		Fat Pad		Breast Fillets		Tenders		Total Breast		Wings		Thighs		Drums	
Item	kg	%	kg	%	kg	%	kg	%	kg	%	kg	%	kg	%	kg	%
Unchallenged –1.05	2.421	75.49	0.032	1.00	0.721	22.38	0.140	4.36	0.861	26.74	0.249	7.75	0.309	9.61	0.299	9.31
Unchallenged – 1.29	2.434	75.71	0.032	0.98	0.722	22.41	0.143	4.45	0.865	26.85	0.252	7.84	0.317	9.87	0.299	9.33
Challenged – 1.05	2.395	75.34	0.031	0.99	0.703	22.11	0.138	4.34	0.841	26.45	0.247	7.81	0.310	9.77	0.298	9.41
Challenged – 1.29	2.394	75.72	0.029	0.91	0.705	22.23	0.136	4.30	0.840	26.53	0.249	7.88	0.315	9.97	0.298	9.43
SEM	0.021	0.129	0.001	0.028	0.009	0.179	0.002	0.050	0.010	0.182	0.003	0.042	0.005	0.120	0.003	0.050
Factorial main effect of Challenge																
Unchallenged	2.428	75.60	0.032	0.99	0.722	22.40	0.142[a]	4.41	0.863[a]	26.80	0.251	7.80	0.313	9.74	0.299	9.32[b]
Challenged	2.395	75.53	0.030	0.95	0.704	22.17	0.137[b]	4.32	0.841[b]	26.49	0.248	7.85	0.313	9.87	0.298	9.42[a]
SEM	0.021	0.129	0.001	0.028	0.009	0.179	0.002	0.050	0.010	0.182	0.003	0.042	0.005	0.120	0.003	0.050
Factorial main effect of Arg ratio																
1.05	2.408	75.42[b]	0.032	1.00	0.712	22.25	0.139	4.35	0.851	26.60	0.248	7.78	0.310	9.69	0.299	9.36
1.29	2.414	75.72[a]	0.031	0.95	0.714	22.32	0.140	4.38	0.853	26.69	0.251	7.86	0.316	9.92	0.299	9.38
SEM	0.021	0.129	0.001	0.028	0.009	0.179	0.002	0.050	0.010	0.182	0.003	0.042	0.005	0.120	0.003	0.050
Factorial *P*-values																
Main effect of Chall.	0.123	0.587	0.081	0.191	0.080	0.217	***0.027***	0.113	***0.045***	0.104	0.352	0.277	0.925	0.275	0.722	***0.046***
Main effect of Arg	0.766	***0.031***	0.147	0.111	0.906	0.687	0.855	0.683	0.891	0.615	0.430	0.069	0.171	0.066	0.943	0.646
Challenge × Arg	0.726	0.549	0.246	0.254	0.990	0791	0.252	0.217	0.826	0.930	0.791	0.812	0.695	0.797	0.794	0.951

[a-b]Values in columns not sharing the same letter are significantly different ($P \leq 0.05$).

[1]Values are least square means of 8 replicate pens per treatment with 12 birds per pen.

[2]Birds were challenged by oral gavage with a 10-fold dose of a live coccidiosis vaccine at 15 d as well as a 10^7 and 10^6 CFU dose of *C. perfringens* at 19 and 20 d, respectively.

[3]Individual live weights were taken the morning of processing (40 d).

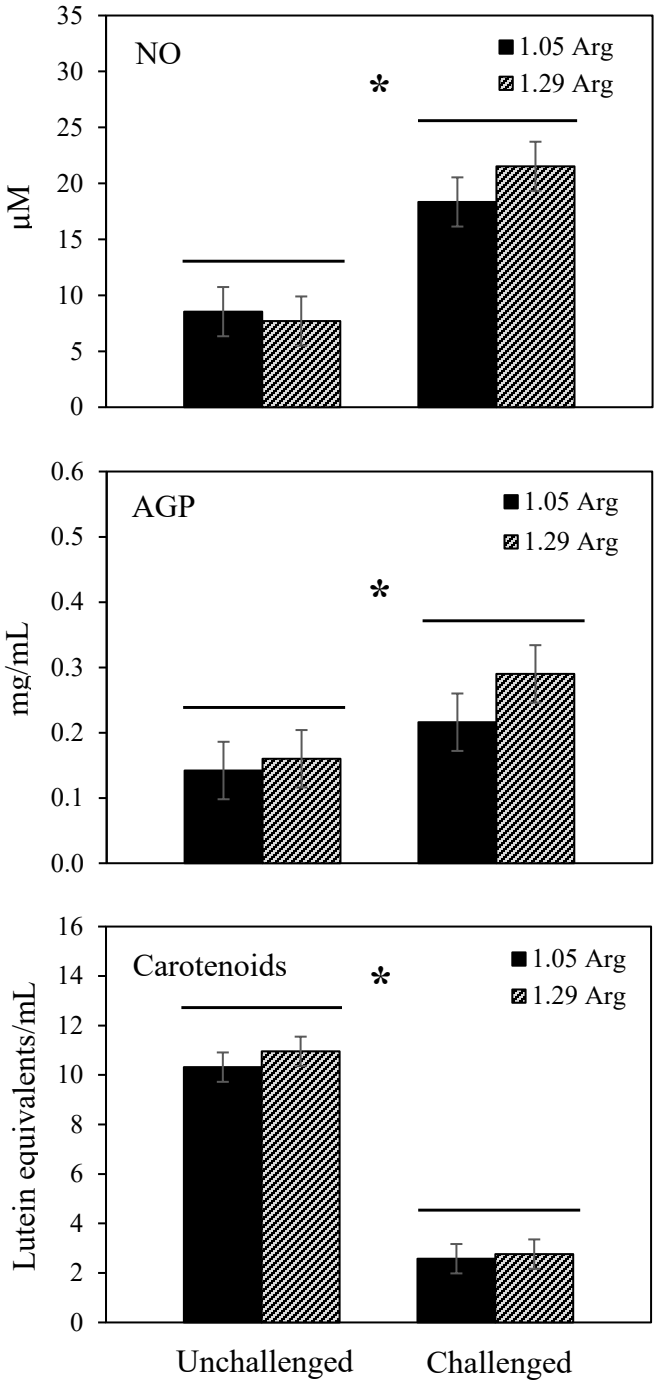

Figure 5.1. Plasma concentrations of nitric oxide (**NO**), alpha-1-acid glycoprotein (**AGP**), and carotenoids at 22 d of Ross 708 male broilers fed increasing concentrations of Arg from 11 to 39 d of age. Birds were challenged by oral gavage with a 10-fold dose of a live coccidiosis vaccine at 15 d as well as a 10^7 and 10^6 CFU dose of *C. perfringens* at 19 and 20 d, respectively. Plasma concentration was significantly increased by challenge for NO ($P < 0.0001$) and AGP ($P = 0.027$), but decreased by challenge for carotenoids ($P < 0.0001$). There were no significant trends

observed with increasing Arg within the challenge group fed the dArg:dLys titration (0.81 to 1.53 dArg:dLys) for any of these measurements ($P > 0.05$; data not shown). Plasma NO was determined by colorimetric assay (Cayman Chemical Company, Ann Arbor, MI), AGP by chicken AGP sandwich ELISA (Life Diagnostics, West Chester, PA), and carotenoids by colorimetric protocol under yellow light (Allen et al., 1996). Values are least square means of 8 replicate pens per treatment with 1 sampled bird per pen.

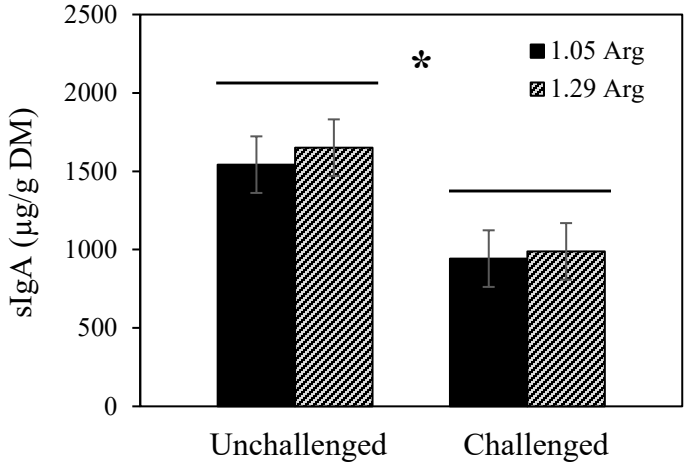

Figure 5.2. Ileal secretory IgA (**sIgA**) concentration (µg per g digesta) in ileal digesta from Ross 708 male broilers at 22 d fed increasing concentrations of Arg from 11 to 39 d post-hatch. Birds were challenged by oral gavage with a 10-fold dose of a live coccidiosis vaccine at 15 d as well as a 10^7 and 10^6 CFU dose of *C. perfringens* at 19 and 20 d, respectively. sIgA concentration was significantly reduced by challenge ($P = 0.001$), but there were no significant trends observed with increasing Arg within the challenge group fed the dArg:dLys titration (0.81 to 1.53 dArg:dLys) ($P > 0.05$; data not shown). sIgA secretion was determined using a chicken IgA double antibody sandwich ELISA kit (Abnova, Taipei City, Taiwan). Values are least square means of 8 replicates pens per treatment with 2 sampled (pooled) birds per pen.

VI. CONCLUSION

As a large majority of the poultry industry is formulating broiler feeds to the 4th limiting amino acid (**AA**), there is a continuing need to understand the optimal requirements of further limiting AAs (arginine, valine, isoleucine), not only to maximize performance but also other biological functions. As arginine (**Arg**) is a "functional AA" with various roles in immunity, thermoregulatory vasodilation, and gastrointestinal tract health, it has become imperative to establish if Arg requirements to optimize these secondary functions differ from those traditionally used to maximize growth performance and meat yield.

Experiment 1 (Chapter 3) established that the dietary dArg:dLys ratio clearly influences the acute (0 to 48 h) inflammatory response following intradermal lipopolysaccharide injection. Most notably, plasma nitric oxide (**NO**) concentration was linearly increased by increasing dietary dArg:dLys at 6 h post-injection, indicating that at this time point Arg was a limiting nutrient for NO production even at the highest dArg:dLys ratio of 1.33. Possibly, this early NO response is responsible for some of the other dose-dependent effects attributed to Arg in this experiment, although this remains to be proven. The additional effects of Arg included an increase in the overall amount of heterophils in the injection-site tissue and peripheral blood, a higher heterophil to lymphocyte ratio in the tissue and blood, and dose-dependent increases in the relative mRNA expression of various pro-inflammatory cytokines and chemokines in the tissue.

Experiments 2 and 3 (Chapter 4) confirmed that dietary dArg:dLys ratio can have positive effects on broiler performance and welfare during conditions of cyclic heat stress (**HS**). In fact, finisher-phase feed conversion ratio (**FCR**) continued to decrease linearly with increased

Arg up to the highest dArg:dLys ratio of 1.40, precluding identification of a break point for the estimation of a requirement. Processing weights and yields were also influenced by higher Arg ratios, but the improvements differed between genotypes. Furthermore, these experiments indicated that dietary dArg:dLys ratio can influence core body temperature during HS in a dose-dependent manner; however, it is important to note that this response was achieved in only one of two genotypes, highlighting the sensitivity of this response to alternative factors.

Finally, experiment 4 (Chapter 5) confirmed that dietary dArg:dLys ratio can also have effects on broiler performance during conditions of enteric challenge with *Eimeria* spp. and *Clostridium perfringens*. Specifically, dArg:dLys ratio quadratically influenced cumulative BWG and FCR of challenged birds, although FCR during the challenge recovery period (7 to 14 d post-challenge) continued to improve linearly up to the highest dArg:dLys ratio of 1.53. In this experiment, dietary dArg:dLys was not found to change plasma levels of NO or AGP. However, regardless of challenge, 1.29 dArg:dLys improved chilled carcass yield compared to birds fed 1.05 dArg:dLys.

Overall, the experiments in this dissertation confirm that Arg is a dynamic AA with secondary metabolic functions critical for optimizing many protective and reparative responses in broilers during conditions of immunological, environmental, and pathological stress. Consistently, many of these responses were positively modulated by dArg:dLys ratios higher than current recommendations. Perhaps this is a contributing factor as to why FCR is often still responsive at dArg:dLys ratios beyond those that have plateaued for other performance and yield measurements. Further research is still needed to confirm the specific mechanisms by which Arg supplementation exerts its positive effects, as they may differ depending on the condition.